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Phagophore membrane connections and RAB24 in autophagy

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ACADEMIC DISSERTATION

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“Where did you come from,
where did you go”
-Rednex

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen EL. Monitoring autophagy by electron microscopy in Mammalian cells. Methods Enzymol. 2009;452:143-64.

II Ylä-Anttila P†, Vihinen H†, Jokitalo E, Eskelinen EL. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy. 2009; 5(8):1180-5.

III Biazik J, Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen EL. Ultrastructural relationship of the phagophore with surrounding organelles. Autophagy. 2015; 11(3):439-51

IV Ylä-Anttila P, Mikkonen E, Happonen K, Holland P, Ueno T, Simonsen A, Eskelinen EL. RAB24 facilitates clearance of autophagic compartments during basal conditions. Autophagy. 2015; 11(10):1833-48.

† These authors contributed equally to this work.
The publications are referred to in the text by their roman numerals (I-IV).
The author's contribution to the publications:

I Contributed to the 3-dimensional modeling in the tomography experiment.

II Performed the experimental work of the immunofluorescence data. Contributed to the 3-dimensional modeling in the tomography experiment.

III Contributed to the experimental design. Performed the experimental work of immunofluorescence data and data analysis. Contributed to the writing of the manuscript.

IV Significantly contributed to the experimental design of experiments. Performed or contributed to most of the experimental work and data analysis excluding cell fractionation, and wrote the manuscript.

Methodological contributions are listed in table 2 in Materials and methods.

ABBREVIATIONS

3-MA	3-methyladenine
ALR	Autophagic lysosome reformation
AMP(K)	Adenosine monophosphate(-activated protein kinase)
Arf	ADP-ribosylation factor
ATG	Autophagy-related
BMP	Bis(monoacylglycero)phosphate
CFP	Cyan fluorescent protein
COG	Conserved oligomeric Golgi
DFCP1	Double FYVE-containing protein 1
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EBSS	Earle's balanced salt solution
EEA1	Early endosome antigen 1
EM	Electron microscopy
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
ES	Embryonic stem cells
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
GAP	GTPase-activating protein
GABARAP	Gamma-aminobutyric acid receptor associated protein
GAS	Group A streptococcus
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTP	Guanosine-5'-triphosphate
HDAC6	Histone deacetylase 6
HeLa	Human cervical carcinoma cell line (Henrietta Lacks)
Htt	Huntingtin
IF	Immunofluorescence
LAMP	Lysosome associated membrane protein
LC3	Microtubule-associated protein 1A/1B-light chain 3
LLPD	Long-lived protein degradation
M6PR	Mannose 6-phosphate receptor
MACF 1	Microtubule actin crosslinking protein 1
MCS	Membrane contact site
MPR	Membrane proximal region
mTOR	Mammalian/mechanistic target of rapamycin
mTORC1	Mammalian/mechanistic target of rapamycin complex 1
MVB	Multivesicular body
NPC1	Niemann-Pick disease, type C1 protein
NRK	Normal rat kidney cells
NSF	N-ethylmaleimide sensitive factor

ORP	Oxysterol-binding (OSBP)-related proteins
PAS	Pre autophagosomal structure
PE	Phosphatidylethanolamine
PI3P	Phosphatidylinositol 3-phosphate
PQ	Polyglutamine
RAB	Ras-related protein in brain
Raptor	Regulatory-associated protein of mTOR
REP	RAB escort protein
RGGT	RAB geranylgeranyltransferase
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
SM protein	Sec1/Munc18-like protein
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNX18	Sorting nexin 18
SQSTM1	Sequestosome 1
STX17	Syntaxin 17
TEM	Transmission electron microscopy
TFEB	Transcription factor EB
TFRC	Transferrin receptor 1
TGN	Trans-Golgi network
TMD	Trans membrane domain
ULK	Unc-51-like kinase
VAMP	Vesicle-associated membrane protein
VMP1	Vacuole membrane protein 1
VPS/HOPS	Vacuolar protein sorting/homotypic fusion and vacuole protein sorting
WIPI	WD-repeat protein interacting with phosphoinositides
α-SNAP	Alpha-soluble NSF-attachment protein

SUMMARY

Eukaryotic cells contain membrane-bound organelles to carry out specialized cellular functions. These organelles are inherited in cell division as templates and are augmented by proliferation through production of protein and lipid components by the cell, and the trafficking of these components within the cell. Autophagy is an evolutionarily conserved degradation pathway for cells to maintain homeostasis, produce nutrients for energy production, degrade misfolded proteins or damaged whole organelles, and fight against intruding pathogens. The process of autophagy entails the isolation of cargo by a specialized organelle, called the phagophore, which closes to form a sealed double membrane bound autophagosome. This organelle then undergoes maturation by fusion with endosomes and lysosomes to obtain its degradation capacity. Hence, there are many dynamic membrane modifications that need to take place during the autophagic process. The origin of the autophagic limiting membrane, as well as the clearance of the degradative structures, are yet to be defined.

This study utilized high resolution electron microscopic methods and three dimensional modeling to reveal nanometer scale interactions of phagophores and autophagosomes with other organelles. Immunolabeling techniques at both light and electron microscopy level were utilized to determine which organelles should be sampled at an ultrastructural level. Direct membrane communication was detected between the phagophore and endoplasmic reticulum (ER), (putative) ER exit sites, mitochondria, the Golgi complex, as well as late endosomes or lysosomes. ER was the most frequent proximal organelle to phagophores and autophagosomes and this suggests an involvement of ER in the nucleation process of phagophores.

This study also reveales a role of the small GTP-binding protein RAB24 in the clearance of autophagic structures in cells. Biochemical and microscopic methods in combination showed that RAB24 is needed in the clearance of autophagic structures in nutrient rich conditions i.e. during basal autophagy. RAB24 was confirmed to localize in both of the autophagosome limiting membranes. GTP binding and prenylation of RAB24 were found to be necessary for the targeting of the protein to LC3 positive autophagic structures, whereas tyrosine phosphorylation was less important for this targeting. Electron microscopy revealed that autolysosome-like structures accumulate in cells when RAB24 is silenced, suggesting that it has a role in the clearance of autolysosomes.

1 INTRODUCTION

I.1 Membrane-bound organelles in eukaryotic cells

The eukaryotic cell is compartmentalized into membrane bound organelles that enable the cell to differentiate its functions and to create separation into aqueous spaces that differ from the cytosol. Most cellular processes also occur on membrane surfaces. Membrane bound organelles have to double in cell division in order to meet the demands of the newly formed daughter cells. This is mostly achieved by enlarging existing organelles, and organelle division followed by distribution to both daughter cells. Organelles in general require information from the organelle itself, making cell membrane inheritance essential for maintaining the complex compartmentalization of the eukaryotic cell (Nunnari and Walter 1996). This means that organelles regulate their own biogenesis through intracellular signaling pathways. Different organelles can also influence each other's biogenesis by transcriptional activation, and extracellular signals can regulate organelle biogenesis by modulating the transcription of genes encoding organellar proteins (Nunnari and Walter 1996).

I.2 Organelle formation

Biogenesis of organelles can be thought to happen in a few different ways. Before the cell undergoes mitosis organelles can increase in size and then further be split into two. Fission and fusion is a way to regulate organelle number in a cell. Another model for organelle formation is the maturation model. As the characteristic proteins and lipids are delivered through the secretory pathway to their destination organelles, they are incorporated in transport vesicles. These vesicles are transported through the secretory pathway and may be merged with membranous structures and reformed several times before reaching their destinations. With this transport, the membrane of the transport vesicle is moved from the donor compartment to the acceptor. The essential idea behind the maturation model is that a compartment gains its distinct proteins through membrane dynamics that include the fission as well as fusion of transport or other type of vesicles, and in this process changes in size, shape and composition to mature into another distinct organelle. This can include both retrograde transport to pre-existing equivalent organelles and anterograde transport (Mullock and Luzio 2000, Lowe and Barr 2007). Maturation may also include direct membrane flow from one membrane structure to another via a membrane contact site. De novo formation means constructing something "from scratch". From the point of view of organelle biogenesis this would mean that an organelle is constructed in the place where it is needed. After lipid and protein synthesis in endoplasmic reticulum, these organellar components would be gathered, most likely via vesicular transport or alternatively via non-vesicular transport, to a place of organelle biogenesis. De novo formation could also include local synthesis. Most lipids are produced in the ER, however some glycerolipids are synthesized in mitochondria whereas sphingolipids are synthesized in the Golgi. Hence, the maintenance of the lipid composition of each organelle requires transport of the lipid molecules within the cell. The main ways of lipid exchange between organelles are vesicular transport, soluble lipid transfer proteins and direct membrane contact sites (MCSs). Lipids are also remodeled by specialized enzymes within organelles (Nunnari and Walter 1996). MCSs are important for lipid transport between organelles that are not connected by vesicular transport, for example the ER and mitochondria (Nunnari and Walter 1996, Lahiri et al. 2015). MCSs are currently recognized

as dynamic transport sites that are present in all eukaryote cells. Their common features include enrichment of proteins involved in lipid biosynthesis and trafficking, requirement of tethering factors to form or stabilize the contact between opposing membranes and formation of dynamic transport structures in response to different physiological conditions (Lev 2010). MCSs have been recognized between various intracellular membranes and several lipid transfer proteins have been shown to localize to MCSs (Lev 2010, Lahiri et al. 2015). Oxysterol-binding (OSBP)-related proteins (ORPs) are one group of lipid binding or transfer proteins that have been suggested to be important either in establishing or maintaining the function of MCSs because of their dual targeting property (Weber-Boyvat et al. 2013). ORPs are targeted to the ER but most of them possess domains or other determinants for simultaneous association to other membranes (Vihervaara et al. 2010).

I.3 Membrane traffic and its molecular machineries

The basis of intracellular traffic and the molecular machineries behind membrane modifications have been quite well established. Vesicular carriers shuttle between compartments to deliver proteins and lipids. Even though the molecular machinery involved in vesicle budding, recognition and fusion is often specific for that particular transport step or fusion event, there are groups of proteins that play central and conserved roles in membrane traffic irrespective of their location (Pelham et al. 1995, Wickner and Schekman 2008). The proteins mediating the initial recognition of merging membranes, pull them closer together, and destabilize the lipid-water interface to enable the mixing of lipids from two sources (Jahn et al. 2003). Membrane fusion sites are first recognized by RAB proteins in a process called tethering. Often in fusion reactions active GTP-bound RABs on the donor membrane recruit further effectors for either donor or acceptor membranes to tether these membranes together. After tethering, fusion is initiated by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and Sec1/Munc18-like (SM) proteins. SNAREs combine to form complexes that facilitate the final step in membrane fusion events and are located on both donor (v-SNAREs) and acceptor (t-SNAREs) membranes. SM proteins are soluble, often associate with syntaxin-like SNAREs and are thought to organize SNARE complexes spatially and temporally into productive topological arrangements thus promoting membrane fusion (Jahn et al. 2003, Sudhof and Rothman 2009). After SNARE-catalyzed fusion is complete, the complexes are disassembled in a reaction catalyzed by the ATPase N-ethylmaleimide sensitive factor (NSF) and alpha-soluble NSF-attachment protein (α-SNAP) (Sollner et al. 1993a).

There are several additional factors that are needed in the regulation of membrane fusion in vivo that include proteins called SNARE regulators, and posttranslational modifications such as phosphorylation (Gerst 2003). These factors can regulate membrane fusion events in several ways including SNARE complex formation, binding to formed SNARE complexes for activation or inactivation and also via phosphorylation to regulate binding of SNAREs or SNARE regulators (Gerst 2003, Sudhof 2013). A simplistic view on SNARE assembly is depicted in Figure 1.

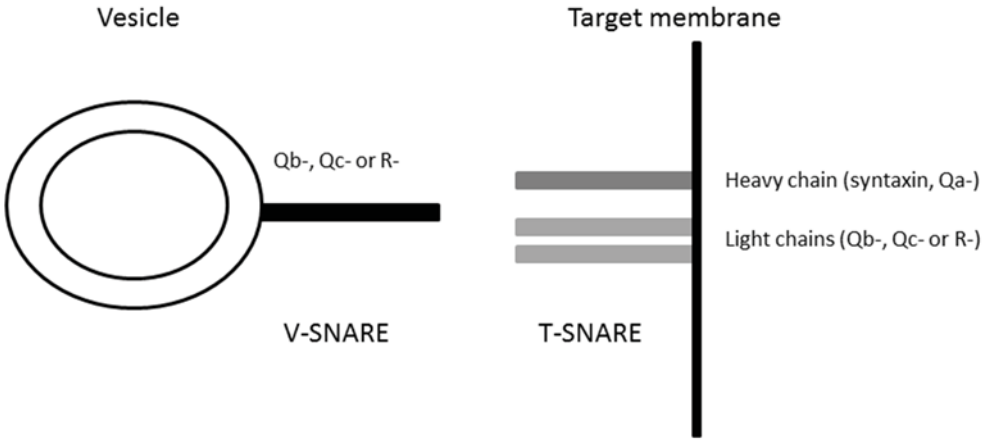

Figure 1. *Arrangement of v- and t-SNAREs. See details in the text.*

I.3.1 SNARE-catalyzed membrane fusion

The SNARE proteins consist of a C-terminal transmembrane domain (TMD) or membrane attachment prenylation, a membrane proximal region (MPR), a helical SNARE-motif with a centrally positioned conserved amino acid residue and an N-terminal domain of varying length and fold (Jahn and Scheller 2006). In addition to the classification into v-SNAREs and t-SNAREs according to the localization to donor vesicles or target membranes, respectively, SNAREs can also be divided based on their structural properties and amino acid sequences. There are three different types of Q-SNAREs that contribute a glutamine (Q) residue to the so called zero ionic layer of the four-helical bundle of the SNARE complex. In addition, there are R-SNAREs that contribute an arginine (R) in the same location. The SNARE complex is arranged so that one helix is contributed from the donor vesicle and this is either a Q-SNARE (Qb or Qc) or an R-SNARE. The remaining three helices, one heavy chain plus two light chains, are contributed by the target membrane and these include always one Qa-type (heavy chain) and two other types of SNAREs (light chains). Qa types comprise the syntaxin sub-family of SNAREs, (Fasshauer et al. 1998, Antonin et al. 2002) whereas Qb SNAREs have amino acid sequence similarity with the N-terminal SNARE-motif of SNAP25 and Qc SNAREs with the C-terminal SNARE-motif of SNAP25. R-SNAREs comprise the VAMP family of SNAREs. All functional SNARE complexes contain one copy of each of the afore mentioned groups; Qa, Qb, Qc and R. Once a so called trans-SNARE complex is formed, the helices make a tight bundle bringing the two membrane surfaces in close enough proximity to fuse. After fusion, the transmembrane regions of the SNAREs are present in the same membrane, now termed cis-complexes. These complexes need to be disassembled for reactivation and consecutive fusions, and this is achieved by the complex formed by NSF and α-SNAP (Sollner et al. 1993b, McMahon and Sudhof 1995, Misura et al. 2000). The SNAREs are then taken away from the newly formed membrane and recycled. A schematic picture of SNARE mediated membrane fusion is presented in Figure 2.

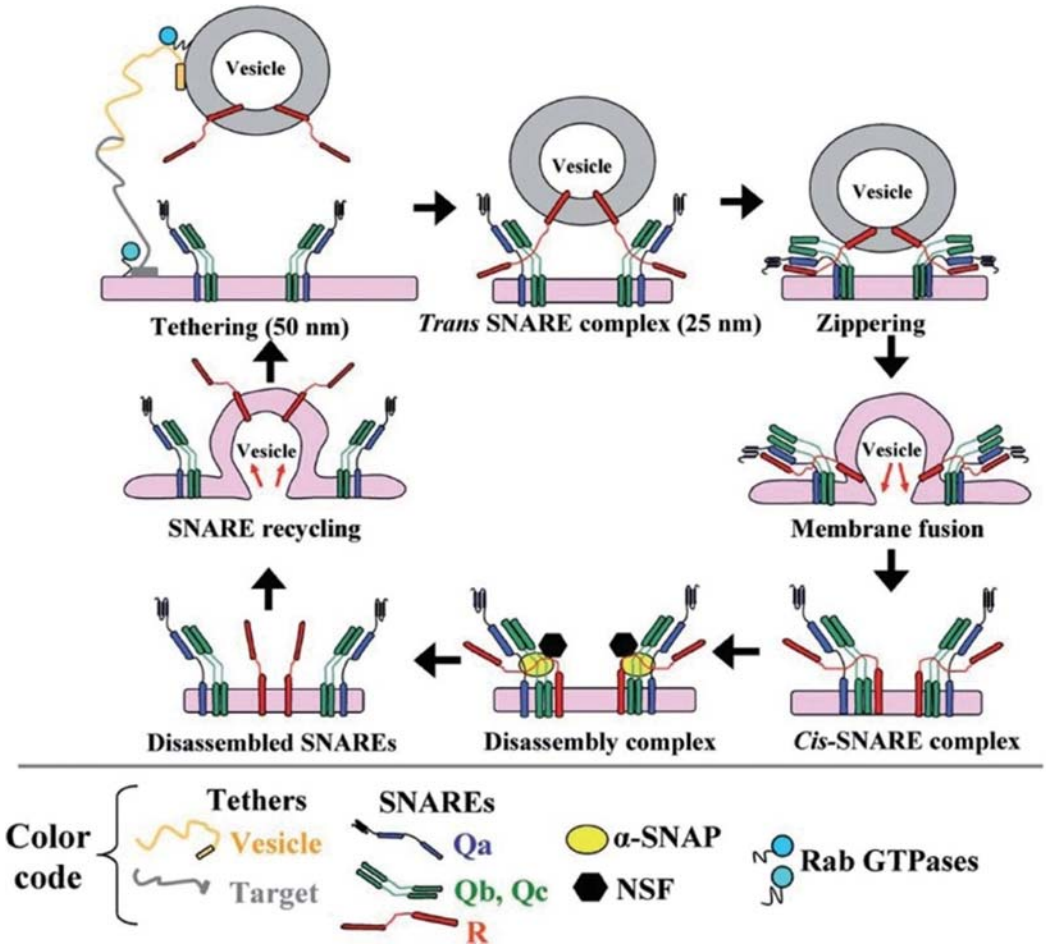

***Figure 2**. The SNARE cycle in vesicle fusion.* (Sehgal and Lee 2011)

I.3.2 RAB cycle

Together with SNAREs, RAB proteins are one of the largest protein families described to be involved in membrane trafficking (Stenmark and Olkkonen 2001). They regulate all steps in intracellular membrane dynamics such as cargo selection, membrane budding from donor compartments, transport along cytoskeletal tracks, docking of vesicles and final fusion events, and therefore are of key importance in accurate intracellular traffic (Stenmark and Olkkonen 2001). RABs consist of a set of so called G-box motifs or nucleotide binding motifs in their N-terminus, two Switch regions that mediate conformation change upon nucleotide binding and a hypervariable domain in the C-terminus that contributes to their localization (Chavrier et al. 1991, Stroupe and Brunger 2000). RABs are synthesized as soluble proteins but are post-translationally modified by the covalent attachment of a geranylgeranyl moiety to their C-terminal cysteines to become membrane-associated on the cytosolic side of intracellular membranes (Pereira-Leal et al. 2001). A simplified schematic representation of RAB structure is presented in Figure 3. Prenylation of RABs is achieved through the action of RAB escort proteins (REPs) and RAB geranylgeranyl transferases (RGGTs) (Pylypenko et al. 2003). REPs bind the nascent RABs simultaneously with RGGTs which catalyze the prenylation reaction

of the C-terminal cysteine residues of RABs, after which they escort the prenylated RABs to their target membrane (Thoma et al. 2001). Membrane-associated RAB proteins can either be activated or retrieved from the membrane. Inactive RABs are bound to GDP. They are retrieved from the membrane by GDP dissociation inhibitors (GDIs) that are able to bind the inactive RAB-GDP and hide their hydrophobic prenyl groups in their hydrophobic groove making them soluble in the cytoplasm (Wilson et al. 1996, Pylypenko et al. 2006, Wu et al. 2007). RAB-GDP dissociation from GDIs and subsequent membrane insertion are achieved by the action of a GDI displacement factor (GDF) (Figure 4) (Dirac-Svejstrup et al. 1997).

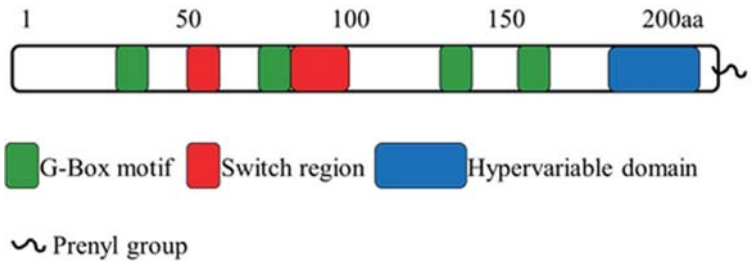

Figure 3. *RAB structure. RAB proteins contain a set of G-Box motifs, two switch regions and a hypervariable domain. RABs are associated with intracellular membranes through a prenyl moiety in their C-terminus.*

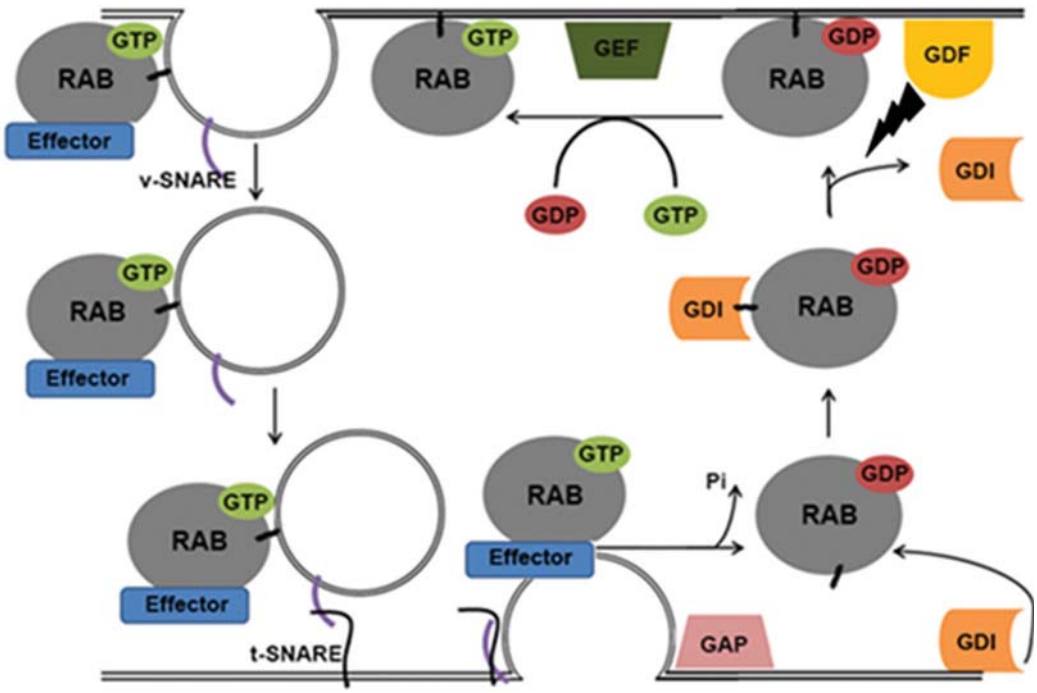

Figure 4. *The RAB cycle. RAB proteins cycle between active, membrane bound form and inactive, cytosolic form. RABs recruit effector proteins while in the active state. GDF detaches the GDI enabling RAB prenyl group insertion to membranes. GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; GDI, GDP dissociation inhibitor; GDF, GDI displacement factor; Pi, inorganic phosphate.*

I.3.3 Molecular switch

The activation status of RABs is determined by the bound nucleotide, GDP or GTP (Dumas et al. 1999). Inactive GDP bound RABs can be activated on membrane surfaces by the action of GDP-GTP exchange factors (GEFs) (Bos et al. 2007). While in the active GTP bound form, RAB proteins are able to recruit effectors to different vesicular traffic steps. The GTP hydrolysis function of RABs is controlled by the GTPase activating proteins (GAPs) (Bos et al. 2007). GTP hydrolysis leads to the inactivation of the RAB and subsequently, RAB-GDP can be reassociated with GDIs and retrieved from the membrane (Ullrich et al. 1993). Unlike REPs and GDIs, GEFs and GAPs show more specificity for their target RABs (Figure 5) (Bernards 2003, Bos et al. 2007).

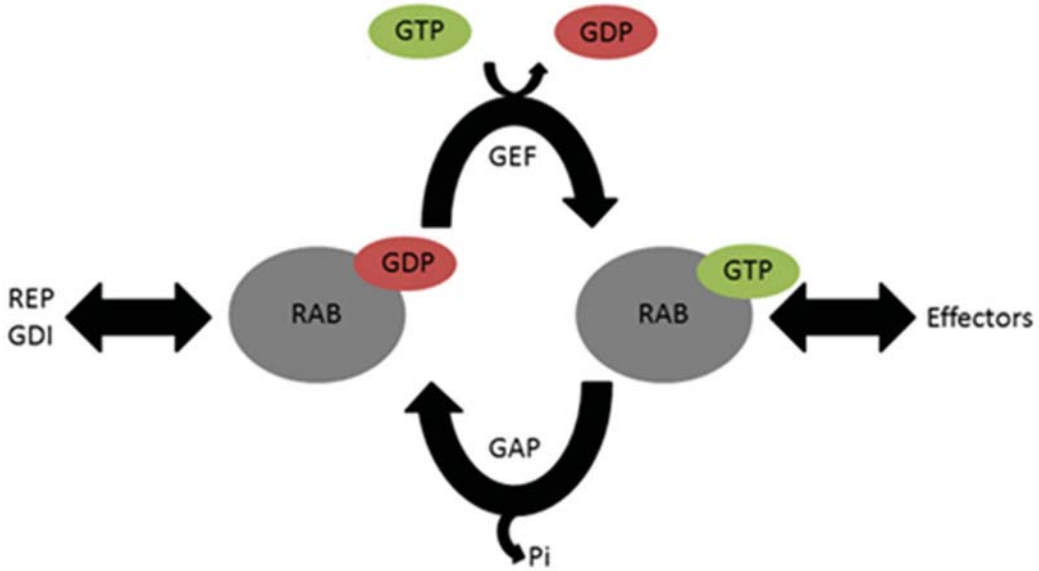

Figure 5. *RAB protein activation is regulated by GTP exchange factors (GEFs) and GTPase activating proteins (GAPs). RABs are active while bound to GTP and inactive while bound to GDP. GDI, GDP dissociation inhibitor; REP, RAB escort protein; Pi, inorganic phosphate. Adapted from Stenmark and Olkkonen 2001.*

I.3.4 The effectors of RABs

RABs control membrane traffic through recruitment of effector proteins that perform different functions during each step of the membrane trafficking process. RAB effectors are proteins that interact with the active, GTP-bound form of RABs and mediate at least one specific downstream effect. RAB effectors belong to many different protein families and are responsible for the selection and concentration of vesicle cargo, vesicle formation, vesicle transport along actin filaments or the microtubule network, vesicle recognition and fusion. One RAB can interact with several different effector proteins (Bhuin and Roy 2014). GEF-RAB effector complexes stabilize activated RABs on membranes generating a positive feedback loop, which counteracts GAP inactivation and GDI-mediated extraction of RABs from the membranes. The formation of GEF-RAB effector complexes enables the formation of specific membrane domains that can result in the concentration of specific molecules on membrane surfaces (Grosshans et al. 2006). An example of this type of membrane domain formation is the recruitment of phosphatidylinositol 3 kinase

(PI3K) by RAB5 on early endosomes leading to the phosphorylation of phosphatidylinositol to phosphatidylinositol 3-phosphate (PI3P) (Christoforidis et al. 1999).

Many RAB effectors serve a tethering function linking opposing membranes before SNARE pairing (Ho and Stroupe 2015). These tethers can be long range coiled-coil tethers such as the early endosome antigen 1 (EEA1), or short range multi-subunit protein complexes such as the class C vacuolar protein sorting/homotypic fusion and vacuole protein sorting (VPS/HOPS) complex (Markgraf et al. 2007). RABs seem to be linked to each other via their effectors forming so called RAB cascades where one activated RAB recruits an effector which acts as the GEF for a consecutive RAB in the next trafficking step. It was shown that this type of RAB cascade functions in the maturation of early to late endosomes where RAB5 is replaced by RAB7 upon maturation. RAB7 GEF, the class C VPS/HOPS complex was shown to interact with RAB5 and to be required for the RAB5-RAB7 conversion (Rink et al. 2005).

I.4 Autophagy

Autophagy is a cellular waste disposal and recycling mechanism. It is an evolutionarily conserved way for cells to maintain homeostasis, produce energy and building blocks for vital biosynthetic reactions, degrade misfolded and aggregated proteins and even damaged whole organelles, fight against intruding pathogens and participate in the controlled disposal of cell corpses in programmed cell death (Klionsky and Emr 2000, Eskelinen 2005b, Zhang and Baehrecke 2015). The autophagic process functions through the action of lysosomes that bring the degradation capacity to the segregated cellular material. There are essentially three main types of autophagy that all function for degradation of cellular components but in different ways. These types, or pathways, are called macroautophagy (or simply autophagy), microautophagy and chaperone-mediated autophagy (Figure 6).

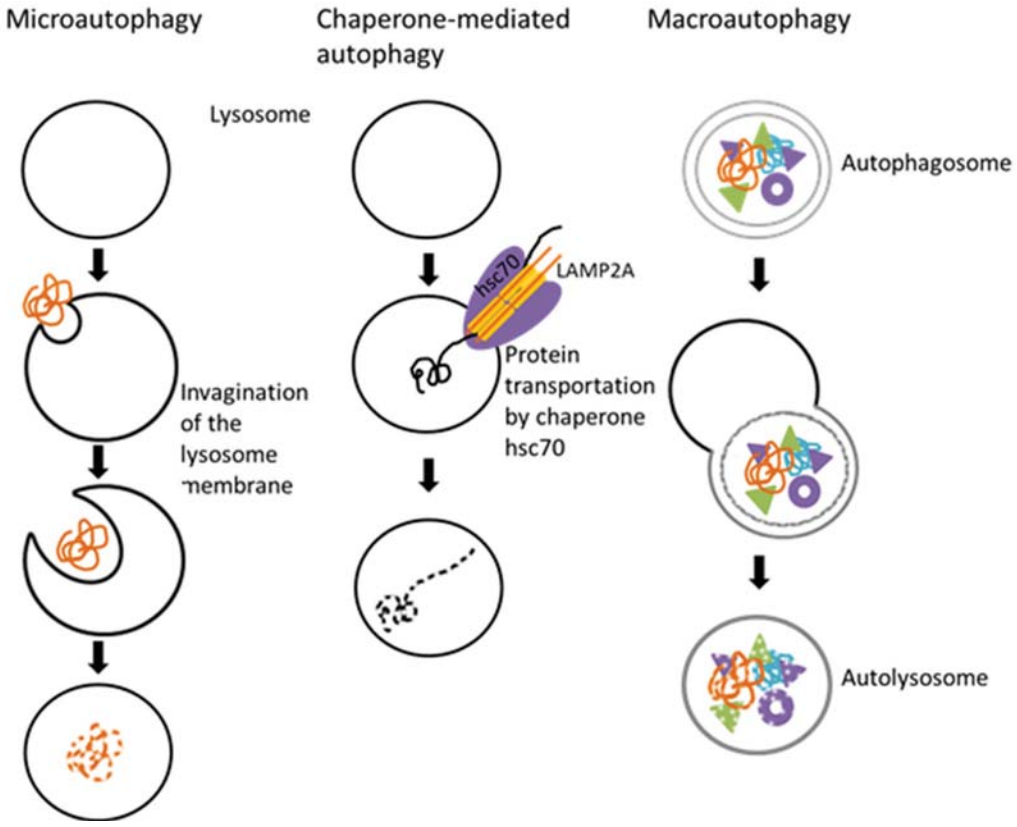

Figure 6. *Different types of autophagy. There are three main types of autophagy: microautophagy, chaperone-mediated autophagy and macroautophagy.*

Macroautophagy is considered the main pathway for the degradation of unused proteins and the only possible way for the cell to degrade whole organelles. Macroautophagy involves the formation of a double membraned vacuole around the degradation bound cargo i.e. the formation of an autophagosome, and the subsequent delivery of the sequestered material for degradation by fusion with endosomes and lysosomes (Mizushima et al. 2002) (Figure 7). Microautophagy occurs by direct inward budding of the lysosomal limiting membrane with the engulfed cargo (Li et al. 2012b). The main difference of these two types of autophagy is the site of cargo sequestration. The third type, chaperone-mediated autophagy, is a very specific transport route to the lysosome where the cargo protein must contain a recognition motif for the receptor protein called lysosomal associated membrane protein type 2A (LAMP2A). The transported protein first binds LAMP2A and after unfolding is transported across the lysosomal membrane with the help of the chaperone hsc70 (Chiang et al. 1989, Agarraberes et al. 1997). After the degradation of autophagic substrates the degraded material is transported back to the cytoplasm through several lysosomal permeases. Each molecule has its transporters in the lysosomal membrane. One example of an amino acid transporter is the seven times lysosomal membrane spanning cystinosin (Gahl et al. 2002), cholesterol is transported with the help of Niemann-Pick disease, type C1 protein (NPC1) (Carstea et al. 1997) and monosaccharides are transported by sialin among other transporters (Verheijen et al. 1999). The importance of specific transport

proteins has been demonstrated by the accumulation of digestion products in lysosomal storage diseases. Thus, autophagy implements the fundamental principal in nature that nothing is wasted and enables cells to recycle its components.

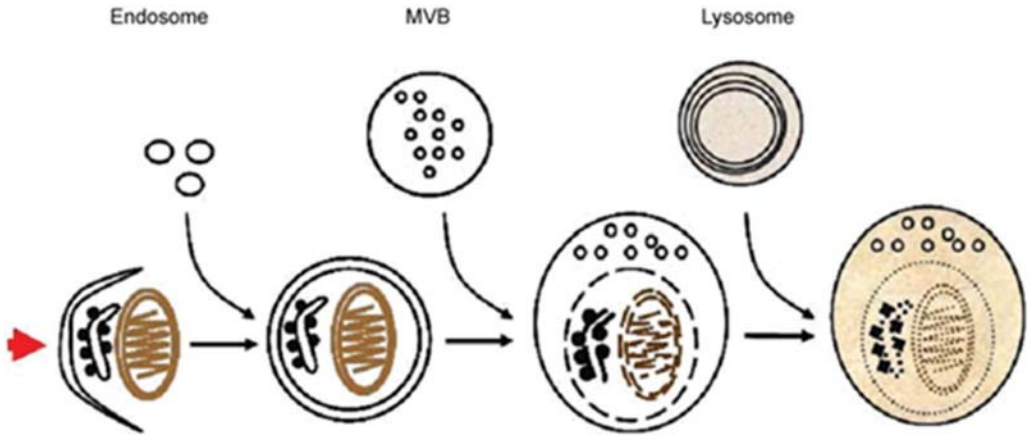

Figure 7. *Schematic view of the macroautophagic process. Cytosolic material is sequestered by a membrane cisternum, called the phagophore or isolation membrane. Phagophore membrane seals to form a closed double-membrane organelle called the autophagosome which then fuses with endosomes and lysosomes to acquire its degradative properties. Degraded material is transported through the lysosomal membrane back to cytoplasm and can be reused by the cell* (Eskelinen 2005b).

After the autophagosome was first described in mammalian cells in the late 1950s by electron microscopy (Clark 1957), new data has been emerging and the steps of the process are relatively well known. However, there are two fundamental questions persisting in the field; namely, where the origin of the phagophore membrane lies and what is the ultimate fate of the autophagic membrane after degradation of the contents has occurred.

I.4.1 The origin of autophagosome membrane

The origin of the phagophore membrane has been puzzling researchers since the transient organelle was described. At the time of their discovery, autophagosomes were observed in electron microscopic images and they could be morphologically distinquished from other organelles. However, there were no molecular markers known for autophagosomes and since the autophagosomal contents reflected the composition of the cytosol, it was challenging to apply the commonly used method of subcellular fractionation to isolate phagophores or autophagosomes (Tooze and Yoshimori 2010). Affirmation of the uniqueness of these membranes came through electron microscopy and cytochemical studies that found it to be relatively protein-poor, as judged by the lack of intramembrane particles observed with freeze-fracture electon microscopy, and heavily stainable with reduced osmium unlike other organelles (Reunanen et al. 1985, Fengsrud et al. 2000). When the techniques for autophagosome isolation developed, the lack of other organelle markers in autophagosome membranes (Stromhaug et al. 1998) gave further support to the assembly model whereby autophagosomes are assembled from constituents at the site of their genesis, i.e., de novo. Despite the uniqueness of the membrane, however, autophagosomes were always observed in the close vicinity of the endoplasmic reticulum

(Eskelinen 2005a). Early morphological studies postulated that autophagic vacuoles derive from (Novikoff et al. 1964) and are continuous with the ER (Novikoff and Shin 1978). At this time the electron microscopy studies were done using serial sectioning of conventional plastic-embedded samples and the three-dimensional models were out of plastic in lack of modern computational methods (Novikoff and Shin 1978). Origin from the ER was also supported by the membrane type of the autophagosome. Autophagic membrane resembles the other thin types of intracellular membranes such as ER, cis-Golgi, nuclear envelope and both mitochondrial limiting membranes. These thinner types of membranes have a physical thickness of about 6-7 nm, whereas the thicker types, such as plasma membrane and lysosomes with more cholesterol and higher membrane protein content, have a thickness of about 10 nm (Arstila and Trump 1968).

I.4.2 Autophagy genes and proteins

Understanding of the autophagic process took a considerable step forward when yeast studies revealed the approximately 30 autophagy related genes (Atg genes) (Tsukada and Ohsumi 1993). Atg genes are needed for the autophagic process to occur and most of them are conserved in mammals (Yang and Klionsky 2010, Mizushima et al. 2011). At present at least five major protein complexes are known to be needed for autophagy: the Atg1-Unc-51-like kinase (ULK1/2) complex; the class III PI3K or Vps34/VPS34 complex which produces PI3P needed for the formation and maturation of autophagosomes; the only known transmembrane protein Atg9/ATG9 and its associated trafficking machinery; and the two ubiquitin like protein conjugation systems Atg12-Atg5 (ATG12-ATG5) and Atg8/LC3-phosphatidylethanolamine (PE) (Figure 8).

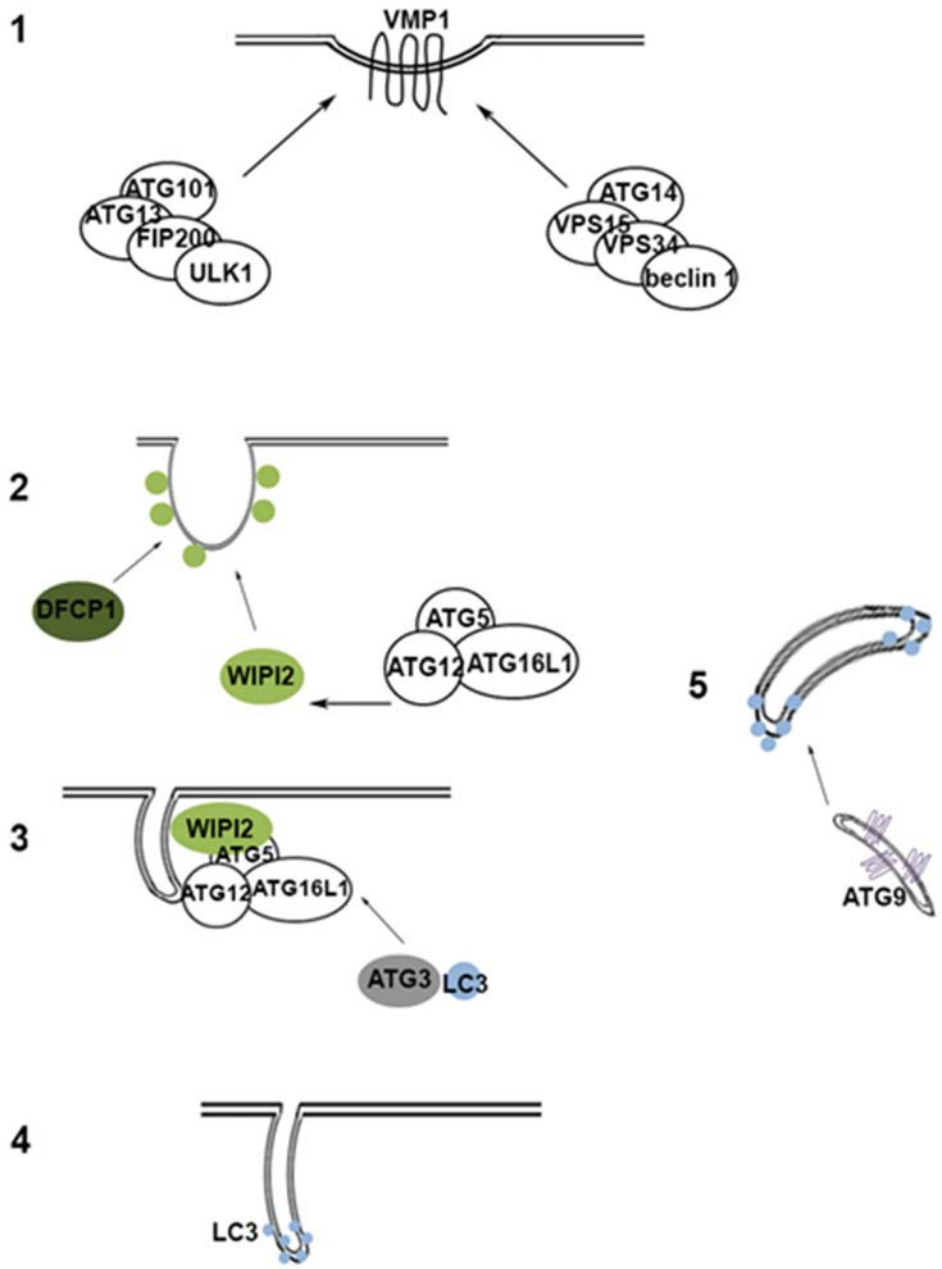

Figure 8. *Model of the recruitment of the protein complexes needed for autophagosome formation. 1. The ULK and PI3K complexes are recruited and PI3K complex is activated by phosphorylation of beclin 1 by ULK1. 2. DFCP1 and WIPI2 bind the lipid PI3P produced by PI3K. 3. WIPI2 recruits the ATG5-ATG12-ATG16L1 complex by interacting with ATG16L1. 4. ATG5-ATG12-ATG16L1 recruits activated LC3 resulting in LC3 lipidation at the membrane. 5. Shuttling of ATG9 contributes to membrane elongation. Adapted from* Carlsson and Simonsen 2015.

Even though the discovery of these major protein complexes has helped in understanding the process, several challenges still remain. Many of these proteins function in the early phase during the regulation of the process, before the formation of the phagophore membrane. Some that

are needed for phagophore formation are initially cytosolic and associate quite transiently with the phagophore only after it has started to form. Even the transmembrane protein ATG9 that is involved in autophagosome biogenesis localizes transiently to the phagophore. However, both genetic and temporal hierarchical studies on the autophagy proteins and their sequence of action have been published (Suzuki et al. 2007, Itakura and Mizushima 2010).

In yeast, Atg17 (part of the Atg1/ULK1/2 complex) is the first protein to be recruited to the pre autophagosomal structure, PAS. Atg17 recruits Atg13 and Atg9, followed by recruitment of Atg1 in an Atg13-dependent manner. It has been shown that the ULK1/2 (mammalian equivalent of Atg1) kinase complex and its regulators are the ones acting most upstream in autophagosome biogenesis forming an initiation site for the process. ULK1 is targeted to the ER subdomains positive for the transmembrane protein called vacuole membrane protein 1 (VMP1) (Koyama-Honda et al. 2013). This is followed by the targeting of the PI3 kinase complex to the initiation site and activation of the PI3 kinase complex by phosphorylation of the complex member beclin 1 by ULK1 (Russell et al. 2013) (Figure 8: 1). Both of these complexes respond to the lack of nutrients and amino acids. ULK1 has been found associated with the mammalian target of rapamycin (mTOR) interacting partner called regulatory-associated protein of mTOR (raptor) (Itakura and Mizushima 2010, Mizushima 2010), which is thought to serve as an amino acid sensor (Sancak et al. 2008). It has also been shown that the negative regulator of autophagy mTOR complex 1 (mTORC1) inactivates ULK1 by phosphorylation of its serine 757 residue by raptor (Kim et al. 2011). This prevents ULK1 interaction and activation by a kinase called AMP activated protein kinase (AMPK).

PI3 kinase triggers the formation of PI3P, which is known to be essential for autophagosome formation (Petiot et al. 2000). This lipid is then recognized by the ER-associated protein DFCP1, as well as WIPI2. DFCP1 is thought to function in the formation of an ER-associated autophagosome nucleation site termed the omegasome (Figure 8: 2). One study found that DFCP1 punctuates on ER membranes upon starvation and recruits the effectors needed for the initiation of phagophore formation (Axe et al. 2008). This live cell imaging study, which showed LC3-positive autophagosomes emerging from DFCP1-positive omegasomes, reintroduced ER as a potential membrane source for autophagosomes. DFCP1 itself is not essential for autophagy.

WIPI1 and WIPI2 are PI3P effector proteins that accumulate at the autophagosome formation site and subsequently on phagophore membranes (Muller and Proikas-Cezanne 2015). WIPI2 has been proposed to promote the development of phagophores inside the omegasomes (Polson et al. 2010) and is responsible for recruiting the ATG12-ATG5 complex by interaction with the ATG12-ATG5 complex member protein ATG16L1 (Dooley et al. 2014) (Figure 8: 3).

The ATG12-ATG5-ATG16L1 conjugation system is needed during the elongation of the phagophore and is located on the outer cytosolic side of the structure (Mizushima et al. 2001). Recycling of ATG16L from the plasma membrane and its interaction with clathrin suggested a role for the plasma membrane in the formation of pre-autophagosome structures. The authors suggested plasma membrane as a possible membrane source for phagophores (Ravikumar et al. 2010). Subsequently, the same group showed that ATG16L-positive precursors undergo homotypic fusion to elongate into pre-autophagosome sructures (Moreau et al. 2011). Further, ATG9 and ATG16L1 were proposed to traffic in distinct vesicles from the plasma membrane

to recycling endosomes, where heterotypic fusion occurs. This was proposed to support autophagosome formation (Puri et al. 2013). Anne Simonsen's group showed that SNX18 is required for recruitment of ATG16L1-positive recycling endosomes to perinuclear region and for delivery of ATG16L1- and LC3-positive membranes to autophagosome precursors (Knaevelsrud et al. 2013b).

The ATG12-ATG5-ATG16L1 complex recruits the other essential ubiquitin-like conjugation system LC3-phosphatidyl ethanolamine (LC3-PE), which is thought to be needed for phagophore membrane elongation as well as the complete closure of the premature structure to form an autophagosome (Fujita et al. 2008). Data from a cell fractionation assay found the ER-Golgi intermediate compartment (ERGIC) as an important factor in the lipidation of LC3 (Ge et al. 2013). LC3 is the most widely used marker for autophagosomes and is attached to both the inner and the outer membrane via its conjugation to the PE lipid. LC3 is present during the formation of the phagophore as well as during autophagosome maturation (Kabeya et al. 2000) (Figure 8: 4). Thus, the LC3 that is trapped inside the autophagosome is degraded together with the cytosolic cargo. LC3 also interacts with autophagosomal cargo via adaptor proteins such as SQSTM1/p62 and NBR1 (Bjorkoy et al. 2005, Pankiv et al. 2007, Kirkin et al. 2009).

ATG9 is a six time membrane spanning protein which shuttles between autophagosomes or other peripheral membranes and the trans-Golgi network and endosomes, and it is thought to serve a membrane delivery function during autophagosome biogenesis (Young et al. 2006) (Figure 8: 5). The shuttling of the protein is dependent on ULK1 and PI3K complex activity and the yeast homolog of ULK1, Atg1, phosphorylates Atg9 (Papinski et al. 2014). Further, this interaction is required for recruitment of the essential autophagy proteins Atg18 and Atg8 to the PAS and for autophagosome membrane formation (Papinski et al. 2014). The trans-Golgi coiled coil protein p230/golgin-245 and its interacting protein, microtubule actin crosslinking protein 1 (MACF 1), are also needed for ATG9 recruitment to phagophores (Sohda et al. 2015). The discovery of ATG9, a membrane protein associated with autophagosome biogenesis raised anticipation about gathering further clues on the membrane source, however, the divergence of the localization of ATG9 did not exactly rule out any existing options, rather the opposite. The main autophagy genes and their functions are summarized in Table 1.

Table 1. *Autophagic genes and their functions. Modified from (Mizushima et al. 2011).*

	Yeast	Mammals	Features	Function
Atg1/ULK complex	Atg1	ULK1/2	Ser/Thr kinase; phosphorylated by mTOR complex 1	In yeast, Atg17 forms a complex with Atg29 and Atg31, and interacts with Atg1 and Atg13 upon starvation to mediate PAS organization. In mammals, ULK1, Atg13, FIP200, and Atg101 form a stable complex irrespective of nutrients and translocate to the autophagosome formation site upon autophagy induction. FIP200 and ATG13 are phosphorylated by ULK1. Atg1, Atg2 and Atg9 are Atg1 kinase targets.

Table 1 cont.

	Yeast	Mammals	Features	Function
Atg1/ULK complex	Atg13	ATG13	Phosphorylated by (m)TORC1	Scaffold for Atg1/ULK complexes.
	Atg17	no known ortholog	Ternary complex with Atg29 and Atg31.	Scaffold for Atg1 complexes.
	Atg11	no known ortholog	Scaffold for the PAS organization in selective autophagy	Recruits fission machinery in mitophagy.
	Atg29	no known ortholog	Ternary complex with Atg17 and Atg31	Needed for the organization of Atg1 complexes.
	Atg31	no known ortholog	Ternary complex with Atg17 and Atg29	Needed for the organization of Atg1 complexes.
	no known ortholog	FIP200	Scaffold for ULK1/2 and Atg13	Stabilizes and phosphorylates ULK. FIP200 and ATG101 are viewed as functional counterparts to the yeast Atg17-Atg29-Atg31 complex without showing sequence similarity.
	no known ortholog	ATG101	Interacts with ATG13	Stabilizes ATG13 expression.
Class III PI3K complex	Vps34	VPS34	PI3K	Produces PI3P in the PAS or on the ER.
	Vps15	VPS15	Ser/Thr kinase; myristoylated, binds VPS34	VPS34 regulator.
	Vps30/ Atg6	Beclin 1	BH3-only protein; interacts with Bcl-2	Acts as a core subunit of the PI3K complex. Phosphorylated by ULK1.
	Atg14	ATG14(L)/ Barkor	Autophagy-specific subunit	Promotes membrane tethering.
	no known ortholog	AMBRA1	Interacts with Beclin 1	Mediates ULK1 dimerization.
Others	Atg2	ATG2A/B	Interacts with Atg18	Needed for autophagosome formation and regulation of size and distribution of lipid droplets.
	Atg9	ATG9L1/2	Multispan transmembrane protein	Delivers membrane to forming phagophores.
	Atg18	WIPI1/2/ 3/4	PI3P-binding proteins	PI3P effector.
	no known ortholog	DFCP1	PI3P-binding FYVE-containing protein	Proposed to function as a platform for autophagosome formation, localizes to omegasomes.

Table 1 cont.

	Yeast	Mammals	Features	Function
Others	no known ortholog	VMP1	Multispan transmembrane protein, interacts with Beclin 1	Localizes to ER, recruits and activates the class III PI3K complex.
Atg12 conjugation system	Atg12	ATG12	Ubiquitin-like; conjugates to Atg5	ATG12-ATG5-ATG16(L) is important for Atg8/LC3-PE conjugation. This complex is present on the outer side of the phagophore.
	Atg7	ATG7	E1-like enzyme	Activates Atg12/ATG12 for conjugation with Atg5/ATG5 and Atg8/LC3 for conjugation with PE.
	Atg10	ATG10	E2-like enzyme	Needed in Atg12/ATG12-Atg5/ATG5 and Atg8/LC3-PE conjugations.
	Atg5	ATG5	Conjugated by Atg12	Forms a complex with Atg12/ATG12 and Atg16/ATG16L1
	Atg16	ATG16L1/2	Homodimer; interacts with Atg5	Interacts with WIPI2 on the phagophore.
Atg8/LC3 conjugation system	Atg8	LC3A/B/C, GABARAP, GABARAPL1/2/3	Ubiquitin-like; conjugates to PE (GABARAPL2 = GATE16)	The formation of Atg8/LC3-PE conjugates and their deconjugation by Atg4 are important for isolation membrane elongation and/or complete closure. Atg8/LC3 is present on both inner and outer membranes of autophagosomes.
	Atg4	ATG4A-D	LC3/Atg8 C-terminal hydrolase; deconjugating enzyme	Catalyses LC3 delipidation.
	Atg7	ATG7	E1-like enzyme (shared with Atg12)	Activates ATG12 for its conjugation with ATG5 and the ATG8 family proteins for their conjugation with PE.
	Atg3	ATG3	E2-like enzyme	Catalyses the LC3-PE conjugation.

Abbreviations: DFCP1, double FYVE-containing protein 1; ER, endoplasmic reticulum; FIP200, focal adhesion kinase family interacting protein of 200 kDa; LC3, microtubule-associated protein 1 light chain 3; mTORC, mammalian target of rapamycin complex; PAS, preautophagosomal structure; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; ULK, Unc-51-like kinase; VMP1, vacuole membrane protein 1; WIPI, WD-repeat protein interacting with phosphoinositides.

An elegant study on the temporal hierarchical recruitment of mammalian ATG proteins to the autophagosome formation site was performed utilizing live cell imaging (Koyama-Honda et al. 2013). In this study ULK1 and ATG5 were simultaneously recruited to a subdomain of ER positive for VMP1. The accumulation of ULK1 and ATG5 was followed by the synchronous appearances of ATG14 and WIPI1 as well as the PI3P-binding omegasome marker DFCP1. A small number of ATG9 positive vesicles were transiently associated with this initiation site. Finally LC3 and SQSTM1/p62 accumulated to the site while the other earlier proteins dissociated. A schematic presentation of the genetic and temporal hierarchy of ATG proteins is shown in Figure 9.

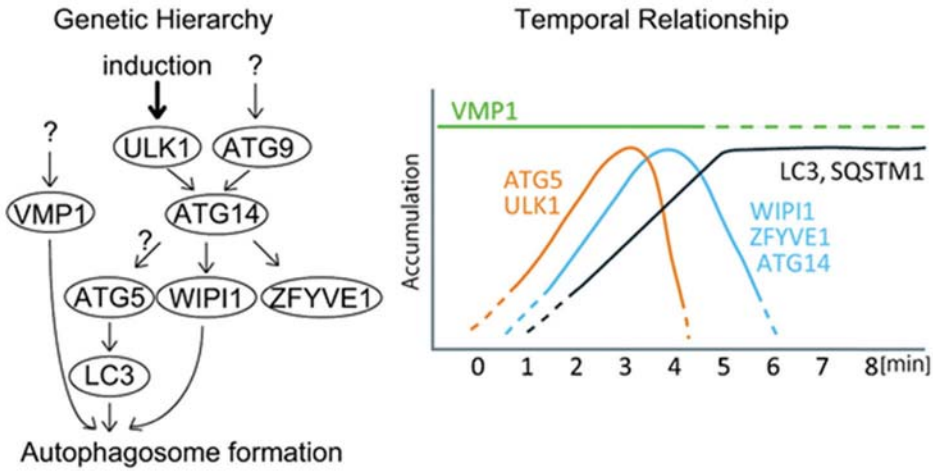

Figure 9. *Genetic hierarchy and temporal relationships of autophagy proteins* (Koyama-Honda et al. 2013).

I.4.3 Regulation of autophagy by nutrients

In addition to containing the degradation capacity of the autophagy process, lysosomes are important in nutrient sensing and signaling pathways involved in metabolism and growth. mTORC1, which controls cell growth, exists on the lysosomal surface and is thought to serve a mechanistic co-regulation between growth and catabolism (Sancak et al. 2010, Laplante and Sabatini 2012). Growth factors, hormones, amino acids, glucose and oxygen activate mTORC1 on lysosome membranes and result in protein synthesis, mRNA and lipid biosynthesis and ATP production (Laplante and Sabatini 2012, Efeyan et al. 2013). Active mTORC1 directly phosphorylates and inhibits the ULK1/2 complex and in this way downregulates autophagy (Ganley et al. 2009, Hosokawa et al. 2009, Jung et al. 2009). If the conditions are not favourable for cell growth, for instance if nutrients are scarce, lysosomes cluster perinuclearly driven by changes in intracellular pH, and this lysosomal positioning deactivates mTORC1 signalling and hence induces autophagy (Korolchuk et al. 2011). Key players in nutrient sensing are the Rag GTPases (Kim et al. 2008, Sancak et al. 2008) that are responsible for mTORC1 recruitment on lysosomal membranes.

It has also been shown that not only is lysosomal positioning important in the regulation of autophagy but lysosomal biogenesis is also tightly linked with autophagy through the transcription factor EB (TFEB) (Settembre et al. 2011). TFEB resides on lysosomal membranes

alongside mTORC1, which in the presence of nutrients inhibits TFEB by phosphorylation. Starvation activates TFEB and promotes its nuclear translocation (Settembre et al. 2012). The Rag GTPase complex, which is responsible for sensing lysosomal amino acids and activates mTORC1, also regulates the nuclear translocation of TFEB. Once in the nucleus, TFEB activates the transcription of genes encoding lysosomal and autophagic proteins.

I.4.4 Basal and induced autophagy

Autophagy is induced by many different stimuli and hence has been predominantly studied under conditions where it is up-regulated. However, autophagy that is independent of nutrient status also exists for maintaining homeostasis. Recent evidence suggests that this kind of non-induced or basal autophagy enforces intra-cellular quality control and is therefore also called quality control (QC) autophagy. Basal autophagy occurs at a low level continuously and is important for cellular maintenance because it degrades for example long-lived proteins, old organelles and protein aggregates; common denominators in age-related disorders such as neurodegenerative diseases. Starvation-induced autophagy and basal autophagy seem to be different in substrate selectivity as well as in regulation and function. While starvation-induced autophagy is inhibited by mTOR kinase, basal autophagy is not affected by mTOR (Yamamoto et al. 2006). The maturation of basal and starvation-induced autophagosomes also differs. RAB7 is known to function during the maturation of starvation-induced autophagosomes but not in basal autophagy (Jager et al. 2004). The histone deacetylase HDAC6, valosin containing protein VCP/p97 and cortactin-dependent actin cytoskeleton promote the maturation of basal but not starvation-induced autophagosomes (Lee et al. 2010, Tresse et al. 2010). These studies also show that the fusion of autophagosomes with lysosomes is actively regulated.

Furthermore, since the main objective of starvation-induced autophagy is to rapidly provide macromolecules for survival under stressed conditions, these autophagosomes logically act in a non-selective fashion. This would allow non-discriminative and effective degradation and recycling of cytosolic contents by starvation-induced autophagosomes. In contrast, basal autophagosomes are thought to have selectivity for protein aggregates and damaged organelles, the degradation of which is mediated by the autophagy adaptor proteins (such as SQSTM1/p62 and NBR1) and through the ubiquitin binding domain of HDAC6. The BUZ domain of HDAC6 is required for its binding to ubiquitinated protein aggregates and it supports autophagosome-lysosome fusion (Lee et al. 2010). HDAC6 is not required for the recruitment of the autophagic machinery to protein aggregates but this is likely mediated by the ubiquitin-binding adaptor protein SQSTM1/p62 which directly binds LC3. In this way basal autophagy could provide a specific and efficient clearance of harmful protein aggregates and damaged organelles, which is especially important for postmitotic cells, like neurons, that are not able to dilute their cytoplasm by cell division. Therefore, basal autophagy serves a function supporting homeostasis and preventing neurodegeneration (Lee and Yao 2010).

I.4.5 Autophagosome maturation and membrane fate

Besides autophagic membrane origin, the ultimate fate of autophagosome limiting membrane is still largely unknown. Being uniquely transient organelles, autophagosomes lose their essential identity via maturation and the convergence of the pathway with the endocytic pathway. Autophagic degradation is known to occur only via lysosomal activity.

I.4.5.1 Lysosome biogenesis and maturation

Lysosome biogenesis itself requires the convergence of the biosynthetic pathway with the endocytic pathway. According to one proposed model, the formation of a lysosome has two requirements; first, the cargo to be degraded from the endocytic pathway and second, the newly synthesized lysosomal proteins, either via ER-Golgi-secretory pathway to the plasma membrane followed by endocytosis, or directly via an intracellular pathway from the trans-Golgi network (TGN) to endosomes (Figure 10) (Saftig and Klumperman 2009).

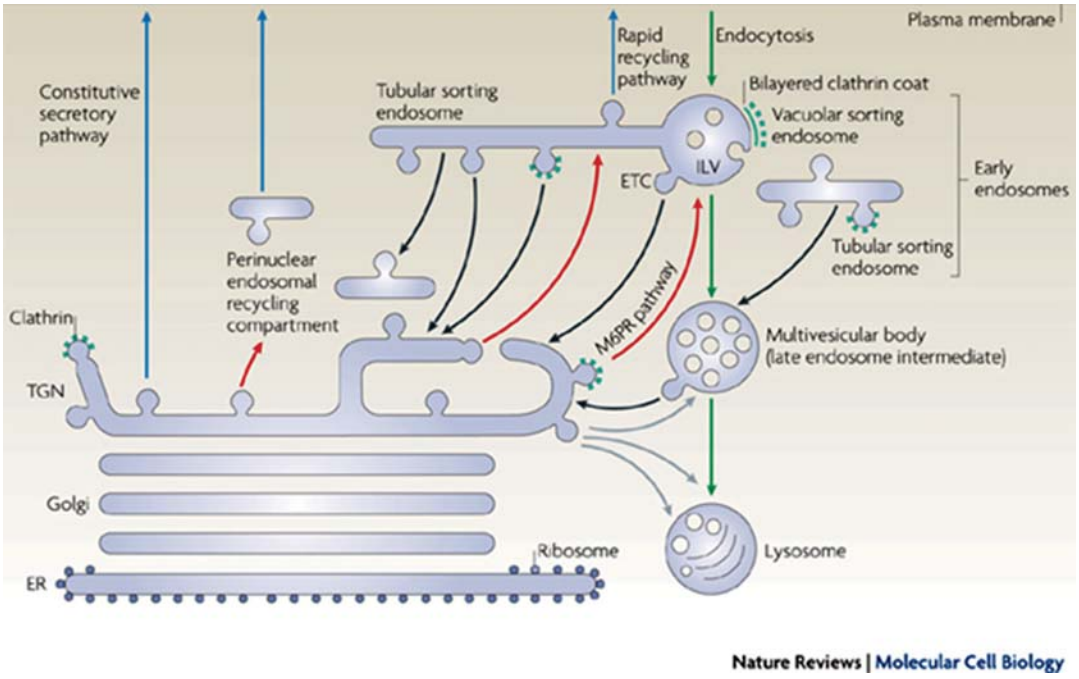

Figure 10. *Lysosome biogenesis and maturation. See details in the text. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Saftig and Klumperman 2009), copyright (2009).*

The acidity of the organelle originates from the activity of the vacuolar ATPase (V-ATPase), a multi-subunit transmembrane complex that is responsible for pumping protons from the cytoplasm to the lumen of endosomes (Lafourcade et al. 2008). The concept of lysosome as an organelle is not straightforward and has been vague since its description. Some view the so-called dense-core or primary lysosomes as being analogous to secretory granules that store acid hydrolases in between fusions with late endosomes, traditional lysosomes being hybrid organelles of fused dense core lysosomes and late endosomes (Luzio et al. 2000). The endocytic pathway originates from the cell surface, when material engulfed from the extracellular space or the plasma membrane is internalized in endocytic vesicles that fuse with early endosomes. Endosomes then mature into late endosomes, also called multivesicular bodies (MVBs). As a result of the maturation, endosomes become increasingly acidic (Lafourcade et al. 2008), which is required for the lysosomal enzyme activity.

There is a significant amount of recycling of different molecules taking place in the early endosome. The morphology of early endosomes can be divided into tubular and vesicular parts, with the former considered to be dedicated for recycling towards the plasma membrane and the latter towards the late endosome. In addition to being increasingly acidified, endosomes grow in size due to homotypic fusions which is partly counterbalanced by the budding of smaller recycling vesicles from the perimeter membrane into the lumen of maturing endosomes. This characteristic morphology has led to the term multivesicular body, which is used alongside the late endosome (Rink et al. 2005). Another model places MVBs as transport compartments that carry material from early endosomes to late endosomes (Gruenberg and Stenmark 2004). As well as morphological differences, endosomes exhibit molecular differences according to their maturation state. The proteins that are used as endosome markers are needed for different membrane trafficking, fusion or sorting processes, as well as recruitment of other proteins needed in dynamic modifications of membrane structures. Early endosomes are marked with proteins such as EEA1, as well as tethering proteins RAB5A and RAB4. Recycling endosomes are rich in transferrin receptor (TFRC) and transferrin, whereas late endosomes or MVBs are marked with mannose 6-phosphate receptors (M6PRs), RAB7 and RAB9 (Russell et al. 2006). Before the convergence of the endocytic pathway and primary lysosomes, the two can be separated by the absence of M6PRs in lysosomes and lysosomal-associated membrane proteins (LAMPs) in early endosomes (Luzio et al. 2000). However, late endosomes are already positive for both M6PRs and LAMPs.

I.4.5.2 Autophagosome maturation

Autophagosome maturation occurs in a similar fashion to endosome maturation with some minor differences. As of yet, no recycling activity in similar scale as in early endosomes has been described in autophagosomes, and the cytoplasmic contents of these structures are destined for degradation. Upon autophagosome closure, the maturation starts by fusion with multivesicular endosomes forming a hybrid organelle termed the amphisome (Berg et al. 1998). This is thought to take place after the recycling events in the endosome have occurred, namely with late, degradation-bound endocytic structures (Yi and Tang 1999). Finally, amphisomes fuse with lysosomes to form autolysosomes (Dunn 1990). Whether the fusion of autophagosomes with endosomes and lysosomes occurs in subsequent separate fusion events or in one fusion event with a hybrid organelle is elusive and might be cell type specific. Autophagosome maturation can be observed by morphology but also by the appearance of lysosomal membrane proteins on autolysosomal membranes, indicationg that they are delivered during the maturation process (Liou et al. 1997, Eskelinen et al. 2002). Maturation of autophagosomes is considered a multi-step process including several fusion events with endo- and lysosomal vesicles. Since lysosomal membrane proteins and enzymes are present in both late endosomes and lysosomes, these proteins can be delivered to autophagosomes during fusion with either of them (Griffiths et al. 1988). It has been suggested that acidification of autophagosomes begins before the delivery of lysosomal enzymes (Dunn 1990). After the fusion events the sequestered material is degraded. Degradative autophagosomes, also termed autolysosomes, are distinct from early autophagic structures by morphology (Eskelinen 2005b). Early autophagic structures, autophagosomes, are surrounded by a double bilayer and contain undigested cytosolic material that resembles normal cytosolic content and may include intact organelles. Autolysosomes have a more dense content where the material is in degradative state. Also the limiting membrane may be a single,

or sometimes a double, bilayer depending on the stage of the degradation process (Eskelinen 2005b).

I.4.5.3 Clearance of degradative structures

After the degradation of the autolysosomal material has occurred, the structures are cleared from the cells. Autolysosome clearance is a poorly understood process. However, recent studies have shown that starvation-induced autolysosomes can be consumed or transformed in a process termed autophagic lysosome reformation (ALR), where new lysosomes are formed by budding from autolysosomal structures and the outer limiting membrane and hydrolytic enzymes are recycled (Yu et al. 2010). Starvation-induced autophagosomes have also been reported to fuse with the plasma membrane allowing their contents to be exocytosed (Ushio et al. 2011).

All forms of autophagic degradation and recycling are dependent on lysosomes and the fate of lysosomal membranes is ultimately linked to lysosomal function: energy metabolism via degradation, secretion, plasma membrane repair and signaling. In degradation, lysosomal properties are maintained with receptor cycling between the Golgi complex and the lysosome. The late endosome and lysosome limiting membrane is also in part turned over by invaginations to form intraorganellar vesicles to degrade cellular membranes. The lipid and protein composition of the intralysosomal vesicles differ from the perimeter membrane so that the internal vesicles are enriched in negatively charged lipids, especially bis(monoacylglycero)phosphate (BMP). At the same time cholesterol, which is abundant in the internal vesicles of recycling tubulovesicular and multivesicular bodies, is mostly excluded from lysosomal internal membranes (Mobius et al. 2003). In order to successfully degrade membranes, some activating proteins or lipids, such as BMP, may be required to allow degradation of lipids (Kolter and Sandhoff 2005). Another way in which lysosomal perimeter membrane is recycled is outward budding in the form of lysosomal reformation. This has been described especially in the context of autophagy where ALR occurs after extended starvation, when amino acids produced by autolysosomal degradation reactivate mTOR, a negative regulator of autophagy. Reactivation of mTOR attenuates autophagy and generates proto-lysosomal protrusions from autolysosomes. Proto-lysosomal budding is clathrin dependent and is initiated by conversion of autolysosome-localized PI(4)P to $PI(4,5)P_2$ by a phosphatidylinositol-4-phosphate 5-kinase (PIP5K1B) (Rong et al. 2012). The generation of $PI(4,5)P_2$ leads to the formation of microdomains on autolysosomal membranes where clathrin is recruited through its adaptor protein AP2. Lysosomal membrane proteins are selectively enriched in these microdomains (Rong et al. 2012). The tubules and vesicles ultimately mature into functional lysosomes (Yu et al. 2010). In lysosomal exocytosis lysosomes secrete their contents by fusing with the plasma membrane. This lysosomal exocytosis also has a crucial role in plasma membrane repair and is one possible fate of lysosomes, maybe also autolysosomes. It has been shown that TFEB regulates lysosomal exocytosis. Overexpression of TFEB enhances lysosomal predocking to the plasma membrane and elevates intracellular calcium concentration which is needed for the fusion of lysosomes with the plasma membrane. TFEB-mediated lysosomal exocytosis promotes cellular clearance and alleviates pathologic storage, restoring normal cellular morphology both in vitro and in vivo in lysosomal storage diseases (Medina et al. 2011).

Lysosomal exocytosis is mostly carried out by secretory lysosomes, which are restricted to certain cell types and are morphologically diverse. They can have dense cores, multilaminar appearance or some unique structures (Blott and Griffiths 2002). Secretory lysosomes have a secretion function; however, conventional lysosomes can also fuse with the plasma membrane as a form of plasma membrane repair. After plasma membrane injury, lysosomes migrate to the damaged site and fuse with the plasma membrane to reseal it (Reddy et al. 2001). Conventional lysosomes are multilamellar or multivesicular by morphology (Blott and Griffiths 2002). The molecular mechanisms and possible differences of these fusion events with the plasma membrane are not clear, however, there are pathologies that specifically affect secretory lysosome function (Blott and Griffiths 2002). Some cells, including platelets and melanosomes, are known to contain both types of lysosomes (Raposo et al. 2001), although this is likely not the case for all cell types.

I.4.5.4 Membrane fusion machineries in autophagy

I.4.5.4.1 Autophagosome formation

There are a multitude of dynamic membrane modifications that need to take place during the autophagic process. An increasing number of membrane fusion machinery proteins are revealed to function in autophagy which is not surprising given all the membrane modification steps required for the process. Several SNAREs, RABs and other membrane fusion proteins are involved in different steps of autophagy. Vesicle-associated membrane protein 7 (VAMP7), syntaxin-7, syntaxin-8 and VTI1B were shown to regulate the homotypic fusion of phagophore precursors and thus to be needed in the autophagosome formation step (Moreau et al. 2011, Moreau and Rubinsztein 2012). Certain SNAREs, namely Sec9p and Sso2p, were shown to be important for autophagosome biogenesis through regulation of the formation of tubulovesicular Atg9 positive structures in yeast. In the absence of these SNAREs, Atg9 vesicles remain small and unable to undergo homotypic fusion (Nair and Klionsky 2011). Also the SNAREs Tlg2, Sec22p and Ykt6p were implicated in this process. Ypt1/RAB1 is known to be needed in ER-to-Golgi transport, but Ypt1 has also been implicated in autophagosome formation through the Atg9-Atg2-Atg18 complex. RAB1 colocalizes with early autophagic structures and loss of RAB1 function decreases autophagosome formation (De Antoni et al. 2002, Zoppino et al. 2010). RAB5 has been shown to work in the same complex with VPS34 and Beclin1 and it is needed for efficient ATG5-ATG12 conjugation and hence autophagosome formation (Ravikumar et al. 2008). RAB33B has been confirmed to interact with ATG16L and enhance the lipidation of LC3 to LC3-II and conversely, the absence of RAB33B was shown to decrease autophagosome formation (Itoh et al. 2008). Further, RAB11 was shown to be needed for autophagosome formation and to colocalize with ULK1 and ATG9 on transferrin receptor (TFRC)-positive recycling endosomes (Longatti et al. 2012). Recruitment of RAB32 to the ER membrane was shown to be necessary for autophagic vacuole formation (Hirota and Tanaka 2009). In yeast, a protein called Sec7 is needed for proper expansion of the phagophore into an autophagosome. Sec7 is a GEF for Golgi-localized ADP-ribosylation factors (Arfs) (van der Vaart et al. 2010). Additionally RAB7, RAB9A and RAB23 have been shown to be involved in the formation of Group A Streptococcus (GAS)-containing autophagosome-like vacuoles (Yamaguchi et al. 2009, Nozawa et al. 2012). RABs and SNAREs in autophagosome formation and maturation are summarized in the Figure 11.

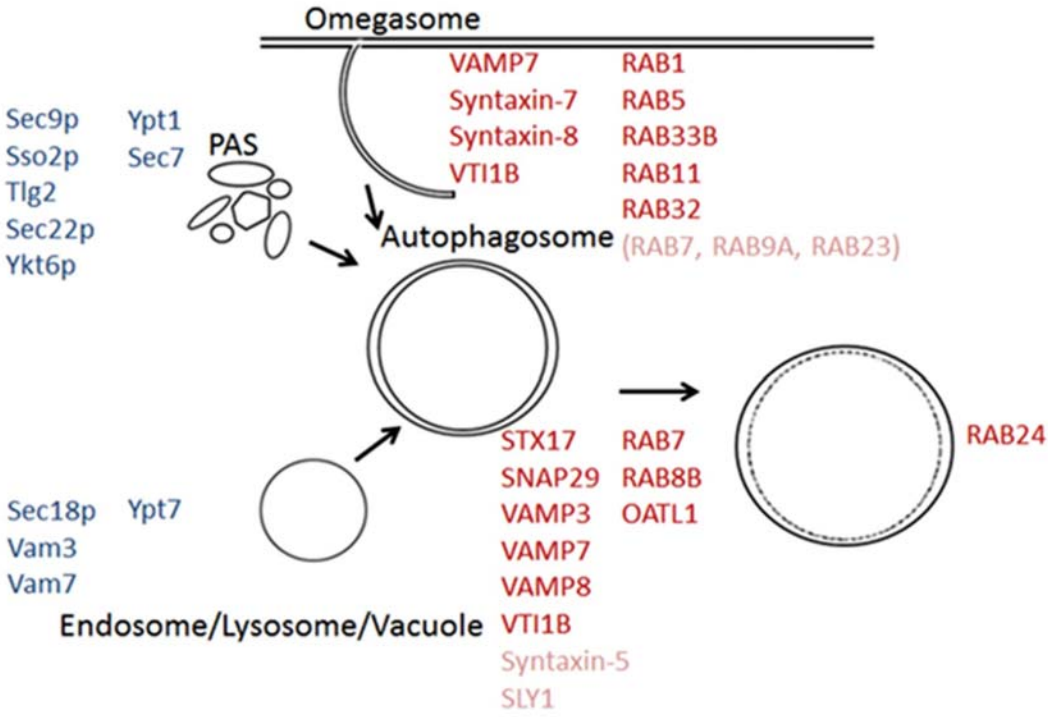

Figure 11. *RABs and SNAREs in autophagy. Yeast proteins are listed in blue and mammalian proteins in red. Lighter color indicates indirect function or function in autophagosome-like vacuoles.*

I.4.5.4.2 Autophagosome maturation and clearance

Autophagosome fusion with lysosomes requires the autophagosomal SNARE STX17, which has been shown to localize to fully closed autophagosomes and to interact with SNAP29 and the lysosomal SNARE VAMP8 (Itakura and Mizushima 2013). All these proteins, as well as VAMP7 and VTI1B, are needed for fusion between autophagosomes and lysosomes (Itakura and Mizushima 2013, Fader et al. 2009, Furuta et al. 2010). Consistently, Snap29 in Drosophila was shown to be needed for the clearance of autophagic structures (Morelli et al. 2014). Recent studies have shown that the autophagy protein ATG14 binds to the STX17-SNAP29-VAMP8 complex and regulates the autophagosome-lysosome fusion (Liu et al. 2015). Autophagosome fusion with multivesicular bodies is regulated by the v-SNARE VAMP3 in an exosome secreting cell line (Fader et al. 2009). Syntaxin-5 and the SM protein SLY1 regulate autophagosome maturation indirectly by regulating lysosome formation (Moreau et al. 2013). The NSF homolog in yeast, Sec18p, has been shown to be needed for the fusion of autophagosomes and the yeast vacuole (Ishihara et al. 2001). Additional factors required for this process are the vacuolar syntaxin homologue Vam3 and the SNAP-25 homologue Vam7 (Darsow et al. 1997, Sato et al. 1998).

Of the RAB proteins, RAB7 has been shown to be needed in autophagosome maturation in several independent studies (Gutierrez et al. 2004, Jager et al. 2004, Ganley et al. 2011, Li et al. 2012a, Hyttinen et al. 2013). RAB7 promotes microtubule plus-end-directed transport and fusion of autophagosomes with lysosomes (Pankiv et al. 2010). RAB8B has been shown to facilitate autophagic elimination of mycobacteria by regulating autophagosome maturation through TANK-binding kinase 1 (TBK-1). In yeast, Ypt7 functions in the autophagosome

fusion to the vacuole (Mayer and Wickner 1997). The absence of RAB25 which is expressed in epithelial tissue has been shown to promote Beclin1 expression and LC3 lipidation (Liu et al. 2012). The GAP of RAB33B, called OATL1/TBC1D25 (TBC1 domain family member 25) has been shown to function in the fusion between autophagosomes and lysosomes through its GAP activity (Itoh et al. 2011). RAB24 has also been shown to colocalize with LC3 (Munafo and Colombo 2002), and deficiency of the RAB24-binding tumor suppressor DRS has been reported to inhibit autolysosome maturation (Tambe et al. 2009). Interestingly, SNAP29, which forms the autophagosomal SNARE complex with STX17 and VAMP8, has been shown to interact with RAB24 and RAB3A (Schardt et al. 2009). In addition, there are other RABs and fusion machinery proteins involved in autophagy whose precise roles are yet to be defined.

II AIMS OF THE STUDY

The aim of this study was to investigate the origin of phagophore membranes and the maturation of autophagosomes. The specific aims were:

1. To investigate which organelles make physical contact with the phagophore

2. To ascertain a functional role for the small GTPase RAB24 in autophagy

3. To propose a specific function for RAB24 in the autophagic pathway

III MATERIALS AND METHODS

A list of the experimental and computational methods and materials used in this thesis is presented in the table below. Detailed descriptions of the methods can be found in the original publications (referenced for each listed method with roman numerals I-IV). A single asterisk * indicates minor contribution by the author, and double asterisk ** a method not applied by the author, in the original publication in question. Methods used in the unpublished results are described below.

Table 2.

Method	Publication
Cell culture	
ES-Atg5$^{-/-}$-GFP-Atg5	II
HeLa	IV
HeLa-Htt-PQ25/65/103-CFP	IV
HeLa-mycRAB24	IV
HeLa-RFP-GFP-LC3	IV
NRK-52E	I, II, III, IV
Cell biology	
Overexpression of proteins	II, IV
siRNA silencing	IV
Construction of stable cell line	IV
Transfection	IV
Sample preparation for microscopy	
Flat embedding**	I
Gelatin embedding**	IV
High pressure freezing and freeze substitution**	III
Immunofluorescence	II, III, IV
Plastic embedding	I, II, III, IV
Post-embedding immunolabeling (Tokuyasu technique)**	IV
Pre-embedding immunolabeling**	III
Thin sectioning**	I, II, III, IV
Tissue embedding**	I
Molecular biology	
Plasmid construction	IV
Biochemistry	
Dot blot filter trap assay	IV
GTP-agarose binding assay	IV
Proteinase protection assay**	IV
Protein measurements (BCA protein assay, filter paper dye-binding method)	IV
Recombinant protein production and purification	IV
SDS PAGE	IV
Subcellular fractionation**	IV
Urea SDS PAGE	IV
Western blotting	IV
Radioisotope methods	
Metabolic labelling for long-lived protein degradation assay	IV

Table 2 cont.

Method	Publication
Microscopy and modelling	
Transmission electron microscopy	I, II, III, IV
3D tomography**	I, II, III
Laser scanning confocal microscopy	II, III, IV
Cellomics CellInsight high content screening*	IV
Serial block-face scanning electron microscopy**	III
Wide-field fluorescence microscopy	IV
Quantitation	
Colocalization measurements	III, IV
Fluorescence area measurements**	IV
Fluorescence intensity per cell measurements*	IV
Fluorescence intensity per organelle measurements	IV
Quantitative electron microscopy*	III, IV
Spot counting	IV
Western blot and dot blot density measurements	IV
Image acquisition and data processing	
Amira, modeling of tomography data*	I, II
AxioImager	IV
CellProfiler	IV
DigitalMicrograph	IV
Excel	III, IV
Image J	IV
Image Lab	IV
ImagePro Plus	IV
IMOD, modeling of tomography data**	III
Las-AF	II, III, IV
Leica Confocal software for SP2 AOBS	II
Photoshop	II, III, IV
Q-Capture Pro	III, IV
Quantity One	IV
SerialEM**	III
Syngene GeneTools	IV
VisionWorks LS	IV

Immunoprecipitation

Cells were lysed in immunoprecipitation-lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl2) supplemented with a protease inhibitor cocktail (Roche # 04693132001). Protein concentrations were measured with a BCA protein assay kit (Thermo Scientific #23228) according to the manufacturer's instructions. Equal amounts of protein (typically 250-1000µg) were used for immunoprecipitation. EZview Red Anti-c-Myc Affinity Gel (Sigma #E6654) was washed, mixed with samples and 1µM γGTP and incubated at +4°C for 1h in a rotator or overnight on ice. Beads were collected by centrifugation and washed three times with lysis buffer supplemented with 1µM GTP before elution of bound protein. Bead pellet was dissolved in 2x Laemmli sample buffer and boiled for 5 min. Samples were analyzed by Western blotting with specific antibodies.

IV RESULTS

IV.1 Three-dimensional electron microscopy revealed connections between the phagophore and its neighboring organelles

The origin of autophagic limiting membrane continues to be one of the open questions in the field of autophagy. Judging from the localization of the proteins and lipids known to be important for the autophagic process, many possible membrane sources have so far emerged including ER, ER exit sites, Golgi complex, plasma membrane and recycling endosomes. Autophagosome biogenesis has also been suggested to take place at the ER-mitochondria contact sites. We used both immunofluorescence staining and three-dimensional electron microscopy to investigate the phagophore morphology in detail, as well as to reveal which other organelles make membrane contacts with phagophores.

IV.1.1 Monitoring autophagy by electron microscopic methods (I)

In this study we have used several different protocols for preparation of cells and tissue samples for electron microscopic analysis. Three of these protocols were described in detail in the methodological paper I. We further discussed the use of different EM protocols for different purposes, such as quantitative or qualitative analysis, as well as methods to perform quantitative EM.

We described resin embedding of aldehyde-fixed cell pellets and of mouse liver tissue for quantitative EM analysis. The former was coupled with postfixation using unbuffered osmium tetroxide (I: Figures 10.4A, B, C and 10.5C) and the latter with postfixation using imidazole-buffered osmium tetroxide (I: Figure 10.5A and B). Both protocols resulted in good preservation of morphology, however, imidazole-buffered osmium which stains unsaturated lipids gave high contrast to autophagosome membranes. This darker staining was especially seen with earlier autophagic structures (I: Figure 10.5A and B) and to a lesser extent with late autophagic structures (I: Figure 10.5B). We also described a protocol for resin flat embedding of aldehyde-fixed cultured cells coupled with postfixation using reduced osmium tetroxide. This protocol was used for detailed, high resolution analysis of a single phagophore with electron tomography. Reduced osmium tetroxide gave good contrast to the phagophore membrane, however, ribosomes that are often used as a marker of the cytoplasmic contents of autophagosomes, had less contrast (I: Figure 10.6A). A three-dimensional model of approximately half of a phagophore was created from tomographic slices of which some are presented in I: Figure 10.6A (I: Figure 10.6B).

We further discussed quantitation methods for EM samples in paper I taken into account two crucial steps in quantitative microscopy, sampling and counting. A quantitation of autophagic compartments in HeLa cells was performed using the unbiased sampling method termed uniform random sampling (presented in Figure 10.1) and point counting protocol (presented in Figure 10.2). Accumulation and clearance of autophagic compartments were estimated in RAB7-depleted cells and controls. The kinetics of the autophagic accumulation was studied using quantitation of samples from three conditions: no treatment (Full), induction with amino acid starvation treatment (AA-) and amino acid starvation followed by a chase in full medium (AA-Full). The amounts of autophagosomes (AC1) as well as late autophagic compartments

(AC2) were quantified. In the absence of RAB7 more late autophagic compartments accumulated in amino acid starvation and the clearance of these accumulated structures was retarded (I: Figure 10.7).

To conclude, we successfully used transmission electron microscopy to obtain quantitative data with methods discussed in detail. Further, we described a method for performing electron tomography to study autophagosomes and phagophores. A three-dimensional model of a phagophore was presented that was produced with the described protocol.

IV.1.2 Autophagosome distribution in cultured mammalian cells (III)

Serial block face imaging scanning electron microscopy (SB-EM) is a method where a resin block containing the sample is mounted onto a platform inside a scanning electron microscope and an inbuilt ultramicrotome within the chamber sections thin, about 40-nm, slices of the block. The electron beam scans the highly contrasted block face after each removed section and images are recorded using a backscatter detector. In this way the images are readily aligned to produce 3-dimensional high resolution data of whole cells. We studied the distribution of phagophores in serum and amino acid starved NRK cells with SB-EM. We observed that the amounts of phagophores and autophagosomes varied greatly between cells, some containing several autophagic compartments while others having none. Further, as far as the distribution of phagophores and autophagosomes inside individual cells was concerned, we observed that a majority of these organelles were located close to the nucleus as well as mid-way along the z axis as opposed to the cell periphery. All observed phagophores located in the close vicinity of ER while the majority of them also had other organelles in their immediate proximity within a distance of about 10 nm (III: Figure 3).

IV.1.3 Many organelles made contacts with the phagophore membrane (II, III)

Since many studies suggest that several organelles or cellular membranes are involved in phagophore formation, we set out to look at the organellar relationships in order to clarify which organelles can be found in the vicinity of phagophores and would thus be the most likely to be able to deliver membrane for their biogenesis. First, we used immunofluorescence staining of endogenous proteins and confocal microscopy to see which organelles were located near forming autophagosomes. The PI3P-binding protein WIPI2 has been shown to localize to phagophores but not to closed autophagosomes (Polson et al. 2010), thus it is a suitable phagophore marker. We performed immunofluorescence double labeling of serum and amino acid starved NRK cells with antibodies against WIPI2 and several other organelle markers and quantified the percentages of phagophores located in close vicinity of each organelle. Close vicinity was considered to be in question if the two labels colocalized in 130-nm optical sections obtained using a confocal microscope. We observed that about 20% of WIPI2 labeled phagophores located close to recycling endosomes positive for TFRC and approximately same proportion located close to the Golgi complex positive for GM130. A little over 10% were located close to late endosomes or lysosomes positive for LAMP1 and about 10% were close to ER exit sites positive for Sec31A (III: Figure 1).

Second, we used pre-embedding immuno electron microscopy to specifically label autophagic structures and visualize their adjacent organelles. Pre-embedding immuno electron microscopy was performed in order to obtain the optimal preservation of morphology while simultaneously utilizing specific labeling of an endogenous marker protein with antibodies. LC3 is present in both phagophores, autophagosomes, and to some extent autolysosomes. However, in electron microscopy autophagosomes and autolysosomes can be distinguished by their morphology: autophagosomes contain morphologically intact cytoplasm and organelles, while autolysosomes contain cytoplasmic material that is morphologically disintegrated. We observed LC3 positive autophagosomes near (20 nm) several organelles. Typically these early autophagic structures were observed next to the ER (59%) and in addition next to one or more other organelles such as mitochondria (22%), Golgi complex (14%), putative ER exit sites or COP II coated vesicles (1%) and endosomes or late endosomes (16%) (III: Figure 2).

Third, we used electron tomography to model phagophores at high, nanometer scale, resolution to see if there was physical contact with phagophore membranes and other organelles. Compared to SB-EM, electron tomography gives a higher resolution, but the sample volume is considerably smaller. Indeed our 3D models revealed phagophore membranes having direct membrane contacts with the ER, putative ER exit sites, mitochondria, endosomes or lysosomes and the Golgi complex (II: Figures 2, 4, 5 and 6; Supplemental Figure 2; Supplemental Videos 3, 5 and 6; III: Figure 4). In one tomogram, we observed a narrow tubular extension from the outer mitochondrial membrane touching the phagophore membrane (III: Figure 5C, D, Supplemental Video S4). In addition, we observed that phagophores were occasionally adjacent to lipid droplets (II: Figures 3A and 5A, Supplemental Figure 1 and Supplemental Videos 1, 2 and 4). Electron tomography also revealed that phagophores frequently have simultaneous membrane contacts with more than one organelle, one of these being the ER.

IV.1.4 Autophagosomes formed in the vicinity of the endoplasmic reticulum (I, II, III)

We used mouse embryonic stem cells expressing GFP-tagged phagophore marker ATG5, as well as NRK cells to quantitatively study the localization of these organelles in relation to the ER. Autophagy was induced by serum and amino acid starvation, and ER was labeled with antibodies against ER markers, either BAP31 or protein disulphide isomerase. Our fluorescence microscopic studies revealed that forming GFP-ATG5 positive phagophores (II: Figure 1A) as well as LC3 positive autophagosomes (II:Figure 1B) were almost exclusively observed close to the ER, often being surrounded by it (II: Figure 1A and B). Rough ER was also often seen close to autophagic structures in conventional TEM images (I: Figure 10.4 and 10.6) so that the limiting membrane of the autophagosome ran in the space between two cisternae of rough ER. ER was estimated to locate either within 20-50 nm from the autophagosome limiting membrane, or inside the autophagosome, in more than half of the cases (II and III: Figure 2) in conventional TEM thin sections.

IV.1.5 ER was the most frequent proximal organelle to phagophores and autophagosomes (I, II, III)

Our 3D reconstructions of phagophores revealed that all of them form contact sites with the ER. The superior resolution provided by electron tomography revealed several new findings. First, we

saw that not only was the ER membrane close to the phagophore but it was also running parallel to it, lining large areas of the phagophore membrane in most reconstructions (I: Figure 6A and B, II: Figures 2 and 3B, Supplemental Video 2; III: Figure 4). Second, in one of our reconstructions, the ER cisterns located inside and outside of the phagophore were connected with each other via a tubular extension (II: Figure 3, Supplemental Video 2). Third, we observed contact points with the phagophore or autophagosome membranes and ER (II: Figures 2-6 and Supplemental Figure S2; III: Figures 4 and 5) most of which were observed between the phagophore membrane and the ER cisterna located inside the phagophore (II). Occasionally we observed that the phagophore membrane was continuous with the ER via tubular extensions (II: Figure 4, Supplemental Video 3). Fourth, the phagophore membrane was not always a full continuous sheet but it occasionally had gaps (II: Supplementary Figure S1B).

To conclude, a total of five tomograms revealed connections between the ER and a phagophore or autophagosome (II: Figures 2-6; III: Figures 4 and 5). These connections were particularly frequent with the ER located inside of the autophagic structures. This finding is in agreement with the results of our immunofluorescence staining and SB-EM, both revealing that phagophores are always located in the close vicinity of the ER.

IV.2 RAB24 in autophagosome maturation and clearance

RAB24 is a small GTPase that has been connected with autophagy in a few studies, but it has not been shown to be necessary for the process. Our goal was to clarify whether RAB24 is actually needed for autophagy and to define the step where it functions.

IV.2.1 RAB24 localization (IV)

IV.2.1.1 Wild type RAB24 localized on autophagosome membranes

We used HeLa and NRK cells to localize RAB24 using immunofluorescence microscopy. Since endogenous RAB24 levels were too low for detection with antibodies, we used transient and stable transfection of myc-tagged RAB24, which we detected using antibodies against RAB24. We also tested that un-tagged RAB24 showed a similar localization, indicating that the myc-tag did not influence our results. Our immunofluorescence data showed that RAB24 colocalized with LC3. Interestingly, this was not entirely dependent on amino acid deprivation. The observed colocalization was obvious if the cells were kept in amino acid free medium for a long time (2 to 4 h) after which the cells contained numerous LC3-positive autophagic structures (IV: Figure 1 and Supplemental Figure 1). However, we were able to observe colocalization of RAB24 and LC3 also in nutrient rich conditions, in those few cells that contained LC3 positive autophagic structures (IV: Figure 1A-C, Figure 2A and Supplemental Figure S1). Quantification of the immunofluorescence signals showed that the percentage of LC3 puncta positive for RAB24 did not significantly change during the serum and amino acid deprivation (IV: Supplemental Figure S1A). However, the amount of RAB24 label per LC3 punctum did increase after 2 and 4 h starvation (IV: Supplemental Figure S1B). In addition the Pearson's colocalization coefficient of RAB24 and LC3 was increased after 1, 2 and 4 h starvation (IV: Supplemental Figure S1D). Immunofluorescence images suggested that RAB24 was mainly localized to the limiting membranes of autophagosomes (IV: Figure 2A). In order to confirm whether RAB24 protein was inside the autophagic structures as cargo, or associated with the limiting membrane, we

performed immunoelectron microscopy using Tokuyasy cryosections. Indeed, RAB24 was localized in the limiting membranes of autophagic structures in both HeLa and NRK cells overexpressing myc-RAB24 (IV: Figure 2B).

To further confirm that RAB24 localized to both the inner and outer limiting membranes of autophagosomes, and to show this for endogenous RAB24 protein, we performed subcellular fractionation of cultured HeLa cells. In addition, we immunoblotted autolysosome and lysosome membranes isolated from rat liver. We found that the endogenous RAB24 protein partially distributed in the same fractions as LC3-II, the membrane bound form of LC3. This was best seen in a continuous OptiPrep gradient, especially when autophagosomes accumulated due to serum and amino acid starvation with added lysosomal inhibitor Bafilomycin A to prevent autophagosome fusion with lysosomes and lysosomal degradation (IV: Figure 2C). Isolated autolysosome and lysosome membranes were positive for the lysosomal membrane protein LAMP1 and LC3-II, as well as the late endosomal/lysosomal protein RAB7. RAB24 was also found in both of these isolated membrane fractions (IV: Figure 2D). A proteinase protection assay of HeLa cell fractions was used to confirm the localization of RAB24 to both the inner and outer limiting membranes of autophagic structures (IV: Supplemental Figure S2). Samples from discontinuous OptiPrep fractionations were incubated either with a serine proteinase TPCK-Trypsin alone, or with TPCK-Trypsin with added NP-40 detergent. Without the NP-40 detergent, free cytosolic proteins and peptides, as well as proteins located on the cytosolic side of vesicles in the samples were digested whereas proteins inside membrane-bound vesicles were protected. Disrupting membranes with the detergent also made vesicle-bound proteins subject to degradation. Similar to LC3-II, RAB24 was partially protected from TPCK-Trypsin degradation, while the cargo protein SQSTM1 was fully protected and EEA1 located on the cytosolic side of endosomes was not protected at all (IV: Supplemental Figure S2). This indicates that endogenous RAB24 was localized on both the inner and outer autophagic limiting membranes.

IV.2.1.2 Targeting of RAB24 to autophagosomes required guanine nucleotide binding and prenylation but not tyrosine phosphorylation

RAB proteins have features that enable their membrane association as well as their function on intracellular membranes, including prenylation of the C-terminal cysteine residues and ability to bind guanine nucleotides. To investigate whether these features are required for RAB24 translocation to LC3 positive autophagic membranes we constructed mutant plasmids. Two mutants that either lacked the C-terminal cysteines (CCΔ) or had the C-terminal cysteines replaced with serines (CC→SS), were confirmed to be prenylation deficient by immunoblotting (IV: Figure 3B). These mutants were expected to be unable to associate with intracellular membranes via the conventional way, through a C-terminal prenyl group. Two of the mutants were expected to be prenylation competent, since they either lacked the unusual histidines (HHΔ) in the C-terminus, or the histidines were replaced by the amino acids SN (HH→SN), thus making the C-terminus resemble that of RAB5 (-CCSN instead of –CCHH). Again, immunoblotting confirmed that these mutants were efficiently prenylated (IV: Figure 3D). Two further mutants, S67L and D123I, were expected to be guanine nucleotide binding deficient. This was achieved by altering the nucleotide binding pocket of RAB24 in such a way that it either did not bind nucleotides at all, or the binding was not stable. Pull down with GTP-beads was used to confirm that the S67L mutant was less efficient in GTP binding than the wild type (WT) RAB24.

The other GTP binding mutant (D123I) did not produce sufficient amounts of soluble protein in the E. coli production system and thus we were not able to confirm its nucleotide binding deficiency. RAB24 also has two tyrosine residues (Y17 and Y172) in its sequence that have been reported to be phosphorylated in cultured cells (Ding et al. 2003). This is unusual for RAB proteins in general; however the biological significance of these phosphorylations is yet to be discovered. We used two mutants, Y17F and Y172F, which had a single tyrosine residue replaced with the phosphorylation incompetent amino acid phenylalanine. Our third phosphorylation deficient mutant, YY17, 172FF, had both of these tyrosines replaced. Our results showed that prenylation deficient (CCΔ and CC→SS) and GTP binding deficient (S67L) RAB24 did not localize to any membranous structures in NRK cells, instead the staining was diffuse indicating soluble cytoplasmic localization (IV: Figures 4-5). Thus, no colocalization was observed between these mutants and LC3 (IV: Figures 4-5 and Supplemental Figure 3). The appearance of these mutant proteins was diffuse both in nutrient rich normal culture conditions and after 4 h serum and amino acid starvation. On the contrary, the prenylation competent mutants of RAB24 (HHΔ and HH→SN) localized to a perinuclear structure, similar to WT RAB24 (IV: Figure 6). This perinuclear structure corresponds to the ER-Golgi intermediate compartment (our unpublished results). The prenylation competent mutants also colocalized with LC3 on punctate structures both in nutrient rich conditions and especially upon 4 h serum and amino acid starvation (IV: Figure 6 and Supplemental Figure S3). Mutants that had one or two tyrosines replaced with phenylalanine (Y17F, Y172F and YY17, 172FF) localized in a perinuclear membranous compartment similar to WT RAB24 (IV: Figure 7 and Supplemental Figures S3-S4). These phosphorylation deficient mutants also colocalized with LC3 similar to WT RAB24 in nutrient rich conditions, although after 4 h serum and amino acid starvation the colocalization was significantly decreased as compared to WT RAB24 (IV: Supplemental Figures S3-S4).

IV.2.2 Effect of RAB24 protein on autophagy (IV)

IV.2.2.1 RAB24 was not needed for autophagosome formation

In order to study whether RAB24 is needed for autophagy, we used small interfering RNA (siRNA) to silence RAB24. Non-targeting siRNA was used as control, and silencing was confirmed by western blotting. After silencing, autophagy was induced by serum and amino acid starvation and autophagic structures were quantified using electron microscopy. Indeed, RAB24 silencing with siRNA revealed an effect on autophagy (IV: Figures 8-9 and Supplemental Figures S5 and S6). Quantitation of autophagic vacuoles in TEM samples was performed using uniform random sampling to omit bias due to subjectivity when taking the images. Surprisingly, the assay revealed significantly more autophagic structures in RAB24 silenced samples compared to the control cells in nutrient rich conditions (IV: Figures 8-9 and Supplemental Figures S5 and S6). However, RAB24 silenced and control cells where autophagy was induced by serum and amino acid withdrawal had no difference in the numbers of autophagic structures (IV: Figure 8 and Supplemental Figure S5). Also, if autophagy was first induced by serum and amino acid removal for 2 h, and then chased out by incubating the cells in normal nutrient rich conditions for 2 h, there was no difference in the numbers of autophagic structures between RAB24 silenced and control cells. From this we could conclude that RAB24 was not needed for autophagosome formation, or the clearance of autophagosomes induced by 2 h deprivation of serum and amino acids. However, autophagic vacuoles accumulated in RAB24 silenced cells under nutrient rich conditions when basal autophagy is expected to operate.

IV.2.2.2 RAB24 was needed for the final maturation step in autophagy or for the clearance of autolysosomes in nutrient rich conditions

We next examined the autophagic flux by using Bafilomycin A, a drug that is widely used for blocking the late phase of autophagy and lysosomal degradation by inhibiting the vacuolar H+ ATPase proton pump. Cells treated with Bafilomycin A accumulate the formed autophagic structures, thus giving information on the rate at which these organelles form. Quantitation of autophagic structures in RAB24 silenced and control cells treated with Bafilomycin A revealed no difference in the numbers of autophagic structures, indicating that the rates of autophagosome formation in these cells were similar (IV: Figure 9). This allowed us to conclude that the difference in the amount of autophagic structures in RAB24 silenced and control cells in nutrient rich conditions was due to hindrance of their clearance. In addition, in this experiment we used both the siRNA pool, composed of four single oligonucleotides, and three of the single oligonucleotides individually, to confirm that we are not observing an off-target effect. The results with the single oligos were similar to the siRNA pool (IV: Figure 9A), confirming that the accumulation of autolysosomes in RAB24 silenced cells was specifically caused by the knock-down of this protein.

IV.2.2.3 Degradative autophagic compartments accumulated in RAB24 silenced cells in nutrient rich conditions

Autophagic structures can be morphologically described immature (ACi) or degradative (ACd) depending on characteristic features described previously in the introduction and results. For ACi, it is often impossible to determine in TEM thin sections whether the structure is a closed autophagosome or a phagophore because of the information lost from the 3D object during cutting of a thin section. However, degradative structures (ACd) possess a degraded cytoplasmic content that is often stained darker than the surrounding cytosol, due to increased contrast of the degraded material. These structures can be identified as closed autophagic compartments even in thin sections. Analysis of the TEM samples revealed that RAB24 silenced cells contain both immature and degradative autophagic structures (IV: Figure 8). However, a considerably larger portion of the accumulating structures were degradative ACd (IV: Figures 8-9 and Supplemental Figures S5 and S6).

IV.2.3 Autophagic clearance in RAB24 depleted cells (unpublished, IV)

IV.2.3.1 RAB24 colocalized with SQSTM1/p62 upon proteasome inhibition

Autophagy is known to increase upon proteasome inhibition to compensate for the compromised protein degradation (Bjorkoy et al. 2005). SQSTM1 (also called p62) serves as a linker to recruit the autophagic machinery to clear ubiquitinated protein aggregates by binding to both LC3 and ubiquitin. Inhibiting proteasomes with MG132 led to the formation of protein aggregates that were labeled with SQSTM1 and colocalized with RAB24 (Figure 12) indicating a possible role for RAB24 in protein aggregate clearance by autophagy. Without proteasome inhibition with MG132 RAB24 did not colocalize with SQSTM1. However, if autophagy was inhibited with the PI3K inhibitor 3-methyladenine (3-MA) simultenously with proteasomal inhibition with MG132, RAB24 was still able to colocalize with SQSTM1 in the aggregates. This indicates that at least the localization of RAB24 to these aggregates was not dependent on autophagosome formation. There seemed to be more and smaller aggregates when both inhibitors, MG132 and 3-MA, were present.

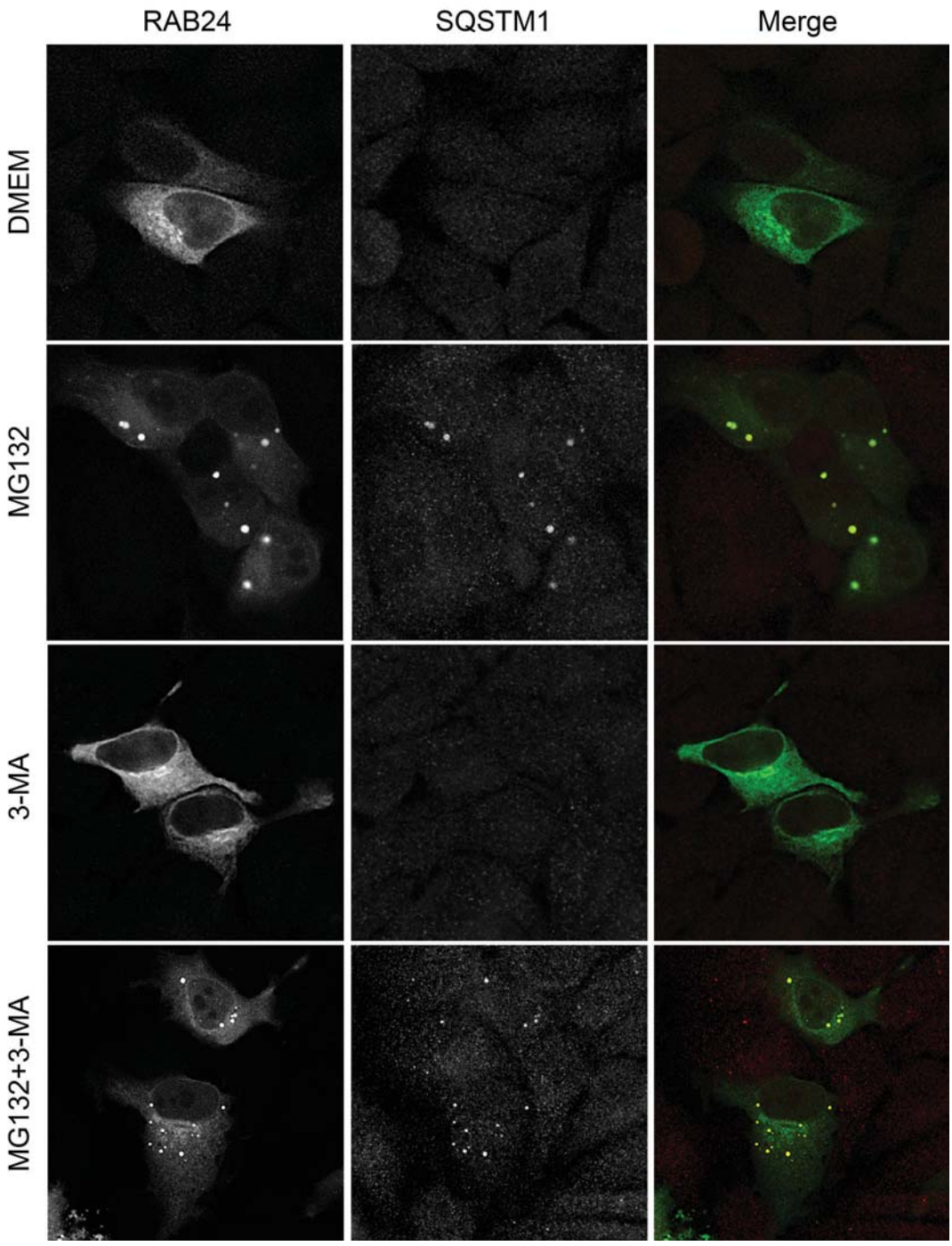

Figure 12. *RAB24 colocalized with the aggregate marker SQSTM1 upon proteasome inhibition with MG132. HeLa cells were left untreated or treated with 10 μM MG132 or 10 mM 3-MA or both for 6 h in full culture medium (DMEM), prepared for immunofluorescence and labeled with antibodies against RAB24 and SQSTM1. Cells pretreated with MG132 showed an altered distribution of RAB24 in SQSTM1 positive aggregates.*

IV.2.3.2 Polyglutamine protein aggregate clearance was defective in RAB24 silenced cells

Since our results showed that RAB24 is needed in autophagy during nutrient rich conditions we decided to perform assays to monitor autophagy in these conditions. Autophagy is thought to serve a homeostasis supporting role in the absence of stress stimuli such as nutrient starvation. It can serve as a protective mechanism in damage control; for example, in addition to the proteasome system, it is known to be the other major mechanism to degrade aggregated or misfolded proteins. We used HeLa cell lines that stably and regulatably express exon1 of htt (exon1htt) encoding a polyQ expansion of 65 residues. The htt peptides contained a CFP-tag to allow their microscopic quantification. Clearance of this htt construct in the HeLa cell line has previously been shown to occur via autophagy (Yamamoto et al. 2006). Two different approaches were used to monitor aggregate clearance in these cells: aggregate counting using fluorescence microscopy and a biochemical filter trap assay to quantify SDS-insoluble protein aggregates. RAB24 was silenced with siRNA and the htt peptide production was inhibited by addition of tetracycline in the growth medium after which the autophagic clearance of the aggregates was allowed for 0, 1 or 3 days. The cells were fixed for microscopic examination, nuclei were stained with DAPI, and the cells were imaged at low magnification. There were more CFP-positive aggregates per nucleus in the RAB24 siRNA transfected cells than in control cells, particularly after 1-day tetracycline treatment when the silencing of RAB24 was most effective (IV: Figure 10C and Supplemental Figure S9). Similar effects were seen with the filter trap assay. Wanker et al. described that large, SDS insoluble protein aggregates are retained in the cellulose acetate membrane while SDS soluble protein in cell extracts is able to penetrate through the fine membrane in a dot blot system (Wanker et al. 1999). More aggregated protein was retained in the cellulose acetate membrane in RAB24 silenced cells as compared to control cells (IV: Figure 10A and B). To conclude, silencing of RAB24 with siRNA delayed htt aggregate clearance (IV: Figure 10 and Supplemental Figure S9). This is likely due to the delay in autophagosome clearance.

IV.2.3.3 Long-lived protein degradation was defective in RAB24 silenced cells

One classic way to analyze autophagic degradation is through measuring long-lived protein degradation (LLPD) since autophagy is a major contributor to the clearance of proteins with a long half-life (Mizushima et al. 2001). This method utilizes metabolic labeling of proteins by incorporating a radiolabeled amino acid. The effect of RAB24 silencing on LLPD was studied in HeLa cells using the release of acid-soluble radioactivity from the cells metabolically labeled with radioactive ^{14}C-valine. We observed that LLPD was slightly delayed in cells where RAB24 was silenced with siRNA. Notably, this effect was significant only in nutrient rich conditions but not during serum and amino acid deprivation (IV: Supplemental Figure S7B).

IV.2.3.4 Autophagosome acidification was not hindered in RAB24 silenced cells

The tandem-tagged LC3 construct, mRFP-GFP-LC3 has been used to monitor autophagosome maturation to acidic autolysosomes, as the GFP fluorescence is lost in an acidic environment while the mRFP fluorescence is more acid resistant (Kimura et al. 2007, Klionsky et al. 2012). To clarify whether the accumulating autophagic compartments in RAB24 depleted cells were acidic, we used a stably mRFP-GFP-LC3 expressing HeLa cell line subjected to RAB24 siRNA transfection and quantified the ratio of areas of yellow (GFP and mRFP fluorescent, neutral)

and red (mRFP fluorescent only, acidic) LC3-positive vesicles. We found no difference between RAB24 silenced and control cells (IV: Supplemental Figure S7A). This result suggests that the autophagic compartments that accumulate in RAB24 silenced cells are acidic, which is also in agreement with the TEM quantification, showing that degradative autophagic compartments (ACd) accumulate in the absence of RAB24 (IV: Figures 8 and 9).

IV.2.3.5 Several markers of autophagic degradation showed no difference upon RAB24 silencing compared to control cells

There are several ways to assess autophagic flux and to evaluate if autophagic degradation is affected. These generally measure the degradation of an autophagic substrate such as polyubiquitinated proteins and SQSTM1. We monitored autophagic flux by immunoblotting with antibodies against these two autophagic substrates, and the autophagosome-associated forms of LC3 (called LC3-II) and another Atg8 homologue called GABARAP. We observed that the level of polyubiquitinated proteins was slightly higher in RAB24 silenced cells, however the difference to control cells was not statistically significant (IV: Supplemental Figure S8A). SQSTM1 levels were similar in control and RAB24 silenced cells (IV: Supplemental Figure S8A). The ratio of cytosolic and membrane bound forms of LC3 and GABARAP, called LC3-I and LC3-II, and GABARAP-I and GABARAP-II, respectively, can also be used to estimate autophagic degradation since the membrane-bound LC3-II and GABARAP-II are degraded in lysosomes together with the autophagic cargo. We found no difference in the LC3-I/LC3-II or GABARAP-I/GABARAP-II ratios in RAB24 silenced compared to control cells (IV: Supplemental Figure S8A). However, we observed slightly higher levels of LAMP1 in RAB24 silenced cells compared to controls (IV: Supplemental Figure S8B). Although considerably large, this difference was not statistically significant in the immunoblot assay, but a statistically significant difference was observed when comparing the intensity of immunofluorescence labeling of RAB24 silenced and control cells with a LAMP1 antibody. RAB24 silenced cells showed a higher LAMP1 labeling intensity per cell than the controls (IV: Supplemental Figure S8B). Taken together, these results indicate that the general autophagic flux is not decreased in RAB24 silenced cells. Further, increased LAMP1 labeling is in agreement with the accumulation of autolysosomes observed by TEM (IV: Figures 8-9 and Supplemental Figures S5 and S6).

V DISCUSSION

V.1 Findings on the forming phagophore

In this study we have chosen to use microscopic methods to examine the forming phagophore and its surrounding organelles, in order to elucidate the membrane source of the phagophore. For visualizing cellular processes, electron microscopy remains superior in the resolution that can be achieved to view fine structural details. There are, however, several attributes to consider methodologically. Despite the superior resolution obtained with electrons, due to 100 000 fold shorter wave length compared with light, the specimen needs to be fixed for EM. This means observing the dynamic cellular processes in a halted state. Still, like many other organelles, autophagosomes were first characterized through EM. As an addition to the high resolution and magnification achieved through EM, a considerable advantage is that the cellular context is readily seen depending on the fixation and staining methods, while in fluorescence microscopy commonly used for live cell imaging, only the fluorescent markers can be observed.

Our morphological studies on phagophores confirmed direct connections of phagophores with the ER, ER exit sites, the Golgi complex, mitochondria and endosomes or lysosomes. We also observed connections of phagophores with lipid droplets. ER was the most frequent proximal organelle to forming autophagosomes and connections between ER and phagophore membrane were present in all constructed tomograms. Further, the inner ER and outer ER lining the autophagic membrane were connected with each other via a tubular extension. These contact sites might represent the omegasome subdomain in the ER membrane. This is supported by a recent study where a detailed morphological study combining correlative light and electron microscopy, immunoelectron microscopy and electron tomography revealed a cluster of thin tubular structures between phagophore edges and the ER (Uemura et al. 2014). Part of these structures were continuous with either the phagophore membrane or the ER membrane or both, and they were positive for the omegasome marker DFCP1 (Uemura et al. 2014). Uemura et al. concluded that these phagophore associated tubular and vesicular structures corresponded to part of the omegasome. Another study describing ER and autophagosome connections was published concurrently with paper II of this study (Hayashi-Nishino et al. 2009), further affirming our findings. As an addition to the observation of the ER having direct contact with the phagophore, the researchers also found GFP-DFCP1 label in ER-phagophore complexes where the phagophore membrane was sandwiched by rough ER membranes. In paper III of this study, we observed clusters of tubules that we identified as part of the ER, close to the open ends of the phagophore. These structures were in direct contact with the phagophore. Thus, these clusters of tubules are very similar to the DFCP1-positive omegasome structures described in Uemura et al. (2014).

In paper III of this study, we observed a subdomain of ER containing coated buds close to the phagophore having contact with it. An emerging model has been described for ER exit sites functioning as the site for autophagosome biogenesis, either by membrane delivery by vesicular traffic, or by acting as a scaffold (Sanchez-Wandelmer et al. 2015). This is supported by numerous studies including the known importance of ERGIC in the lipidation of LC3 and of COPII vesicles in omegasome formation (Zoppino et al. 2010, Ge et al. 2013). Our findings of membrane connection with a putative ER exit site could potentially support both of these new models.

In the present study, we also showed with electron tomography for the first time that autophagosomes can have simultaneous connections with several organelles. This type of observation would be challenging to obtain with fluorescence microscopy or biochemical methods, which might be the reason why it has not been previously described. However, there have been other reports describing a membrane connection between the phagophore and individual organelles. Certain older studies found continuities between autophagosomes and lysosomes observed in serial EM thin sections (Novikoff and Shin 1978, Seglen 1987). These studies are in agreement with our findings that an electron-dense structure inside a late endosome or lysosome is continuous with the phagophore membrane. There has also emerged new data on the importance of recycling endosomes in the phagophore membrane expansion that suggest a possibility of a direct route for lipid transport between these structures (Puri et al. 2013, Longatti et al. 2012, Puri et al. 2014, Knaevelsrud et al. 2013a). These data are in agreement with our findings that as many as 20% of WIPI2 labeled phagophores located close to recycling endosomes positive for TFRC.

Several recent investigations also describe mitochondria as a source of membrane for forming autophagosomes (Hailey et al. 2010, Cook et al. 2014, Ding et al. 2012, Hamasaki et al. 2013, Germain et al. 2011). Mitochondrial outer membrane has been shown to participate in starvation induced autophagosome biogenesis (Hailey et al. 2010). Our finding that a growing phagophore can be in contact with a mitochondrion which simultaneously makes contacts with the ER is intriguing, since phagophores have been suggested to form in the ER-mitochondria contact sites under starvation in mammalian cells (Hamasaki et al. 2013). Therefore, our results in paper III could represent this type of ER-mitochondria contact site.

We also saw a close association of the Golgi cisternae and the phagophore which has not been shown before. The Golgi complex has previously been implicated as a possible membrane source, in particular as a potential source of ATG9 vesicles that are needed for autophagosome biogenesis (Young et al. 2006, Koyama-Honda et al. 2013, Takahashi et al. 2011, Mari et al. 2010, Yamamoto et al. 2012). AP1-dependent clathrin coating at the TGN, as well as exit from the Golgi complex in yeast, has been shown to be needed for the formation of autophagosomes (van der Vaart et al. 2010, Guo et al. 2012). Further, conserved oligomeric Golgi (COG) complex and the genes involved in Golgi-endosome trafficking have been shown to be involved in autophagosome formation and to influence the localization of autophagy proteins Atg8 and Atg9 in yeast (Ohashi and Munro 2010). These studies are in agreement with our findings that Golgi cisternae localize in close vicinity of the phagophore membrane. However, our results showed a direct membrane contact between the phagophore and a Golgi cisternum instead of Golgi derived vesicles. Whether there is vesicular delivery to the phagophore membrane from the Golgi remains to be further confirmed at high resolution EM level.

In the present study, we observed that phagophores were occasionally adjacent to lipid droplets. Recent data suggests that not only are lipid droplets engulfed by autophagosomes providing fatty acids for metabolic needs through lysosomal lipolysis or "lipophagy" (Singh et al. 2009), but they are also important for phagophore formation (Dupont et al. 2014, Shpilka et al. 2015). Enzymes that are needed for lipid droplet formation, particularly triacylglycerol and steryl ester synthesis, are also needed for functional autophagy (Shpilka et al. 2015). It has been suggested that there

may exist a direct kiss-and-run exchange between lipid droplets and forming autophagosomes, whereby lipids are donated to the outer phagophore membrane (Dupont et al. 2014). This can be speculated to contribute to providing curvature by asymmetric loading of lipids to the outer phagophore membrane from the phospholipid monolayer of the lipid droplet or by donating diacylglycerol as a curvature-inducing lipid (Dupont et al. 2014, Deretic 2015). Thus our findings are in agreement with these studies.

V.2 Findings on the effect of RAB24 on autolysosome clearance

Since RAB24 had previously been reported to colocalize with the autophagy markers LC3 and monodansylcadaverine (MDC) (Munafo and Colombo 2002), we wanted to define the precise localization of the protein in autophagosomes and exclude the possibility that overexpressed RAB24 was merely being degraded via the autophagic pathway. Immunoelectron microscopy and cell fractionation confirmed localization of both overexpressed and endogenous RAB24 to both inner and outer autophagosome membranes. Our findings imply that RAB24 is likely to have a function in the autophagic membranes, as opposed to being targeted for autophagic degradation. This conclusion is supported by our unpublished data showing that RAB24 protein levels do not change during serum and amino acid starvation. Further, our preliminary qPCR results showed that RAB24 mRNA was upregulated during starvation, suggesting that increased transcription/translation may compensate for the loss of RAB24 protein due to delivery to lysosomes. Surprisingly, RAB24 was seen on both the inner and outer membranes of the autophagosomes. This was further confirmed with cell fractionation studies combined with a protease protection assay. On most organelles RAB proteins localize on their outer, cytoplasmic surface, since RABs are known to be post-translationally prenylated and cycle between the cytosolic GDP form and the membrane-associated GTP form. However, since autophagosomes are surrounded by a double bilayer, they have a lumen topologically equivalent to the cytosol. Notably, RAB7 known to be needed for autophagosome maturation during amino acid starvation shows a similar localization to both the inner and outer limiting membranes of autophagosomes (Jager et al. 2004). This type of localization suggests that RAB24 is present on the membrane already during the formation of the autophagosome, before it closes, which implies a role for it during phagophore elongation, or in some membrane modifications. However, considering that autophagosomes are able to form in the absence of RAB24 and instead, degradative structures accumulate, it is unlikely that RAB24 is needed during phagophore elongation but rather after the closure of the autophagosome. The localization of RAB24 to both inner and outer autophagosome membranes suggests that its function is not just to facilitate the fusion with endosomes or lysosomes, since that would require localization to the outer membrane only. Some other role seems likely since the accumulated structures in the absence of RAB24 are degradative and LAMP1 positive, hence they have been able to fuse with lysosomes. The localization of RAB24 further suggests that RAB24 is recruited on the membranes during phagophore elongation, rather than being brought to the outer limiting membrane through vesicle fusion. This leaves open the possibility that RAB24 would also have some role in earlier stages of the autophagic process, which were not detected in the autophagy assays used in this study.

RAB24 carries two unusual histidines as the last two amino acids in its C-terminus that have not been described for other RABs (Erdman et al. 2000). This has previously been proposed to contribute to deficient prenylation of the protein, though additional contribution of some other features of the molecule was considered likely, since removal of the histidines did not alter prenylation of overexpressed RAB24 (Erdman et al. 2000). Our results showed that these C-terminal histidines had a minimal influence on the prenylation of RAB24, and further, that prenylation was needed for membrane localization as well as for colocalization with LC3. Due to these histidine residues, the observation that RAB24 localizes also to the autophagosome inner perimeter membrane is especially interesting. The histidine side-chain has a pKa of approximately 6.5 and a relatively small change in pH in the physiologically relevant pH range will lead to a change in its average charge. Thus, change from cytosolic pH of 7.4 to lysosomal pH of 4.5-5.0 would cause a change in the charge of RAB24. This definitely raises questions on the biological relevance of the C-terminal sequence and on possible roles of RAB24 in vesiculation or permeabilization of the autophagosome inner membrane, which is needed to gain access for the cargo to the lysosomal hydrolases.

We also found that nucleotide binding is needed for RAB24 targeting to membranes and for colocalization with LC3 while tyrosine phosphorylation is dispensable. Overexpressed RAB24 has previously been reported to exist mostly in the GTP bound form which is unusual for a RAB protein (Erdman et al. 2000). Interestingly, a mutation in the nucleotide binding switch I region of RAB24 was shown to be the cause of a hereditary neurodegenerative disease in dogs, namely canine ataxia (Agler et al. 2014). Several neurodegenerative diseases have been connected with impaired autophagy (Menzies et al. 2015). Our findings now strongly support the notion that this canine ataxia is connected with decreased autophagic clearance in the affected neuronal tissues.

Overexpressed RAB24 has been reported to be able to undergo tyrosine phosphorylation and the phosphorylated pool of the protein was largely located in the soluble fraction (Ding et al. 2003). It is not known whether the same applies to endogenous RAB24 protein. We observed that tyrosine phosphorylation is dispensable for targeting of RAB24 to LC3-positive structures. However, the relationship of tyrosine phosphorylation and nucleotide binding and hydrolysis may represent a regulatory mechanism for RAB24 targeting and function in certain tissues or under certain conditions.

In this study we show that RAB24 depletion causes accumulation of autolysosomes under basal conditions, while no accumulation was observed under serum and amino acid starvation. Since the amount of autophagic structures in the cell at a given time point is the sum of the rate at which the organelles form and the rate at which they are disappearing, it is essential to know which part of the process is influenced in the case of autophagosome accumulation or loss (Rubinsztein et al. 2009). The function of RAB24 in nutrient rich conditions was supported by our findings that RAB24 colocalized with SQSTM1 under proteasomal inhibition in nutrient rich medium. This enabled us to examine cells in conditions where autophagy is upregulated independent of nutrients. RAB24 was, however, able to colocalize with SQSTM1 under proteasomal inhibition also if the PI3K was simultaneously inhibited by 3-MA. This implies that the localization of RAB24 to protein aggregates was not dependent on autophagosome formation. The immunofluorescence assay did not yield conclusive data about autophagosomal degradation; however the aggregates observed in the presence of MG132 alone compared to

the aggregates observed in the presence of both MG132 and 3-MA, were larger and fewer. This might suggest a hindrance in the processing and degradation of the aggregates. Notably, RAB24 was targeted to SQSTM1-positive aggregates only during proteasomal inhibition with MG132. Further studies revealed that RAB24 also facilitated the clearance of Huntingtin aggregates and long-lived protein degradation in nutrient rich conditions. Previously, increased RAB24 and LC3 mRNA levels were observed in nerve-injured hypoglossal motor neurons of rats. The same study also reported partial colocalization of RAB24 and LC3 (Egami et al. 2005). All these findings support the conclusion that RAB24 likely functions in basal, noninduced autophagic processes, unlike for example RAB7 that functions during the maturation of starvation-induced autophagosomes (Jager et al. 2004).

In addition to defining that RAB24 functions in the clearance of autophagosomes, we further analyzed which part of the clearance process could be affected. We consistently found that the problem upon knock-down of RAB24 was very late during the autophagic process. The autophagic structures that accumulated in RAB24 silenced cells were morphologically degradative and acidic. LAMP1 immunolabeling was also increased in the RAB24 silenced cells, which is in agreement with an accumulation of autolysosomes. If there were a hindrance in the fusion between autophagic structures and lysosomes, the presumption would be that more immature autophagic structures accumulated in the cytosol. Further, markers of autophagic degradation, like ubiquitinated proteins and SQSTM1, did not accumulate in the absence of RAB24 which indicates that degradation proceeds in these accumulating structures. Therefore, we conclude that RAB24 is required at a step downstream of autophagosome-lysosome fusion, for the clearance of autolysosomes.

Interestingly, RAB24 has been reported to interact with proteins of the membrane fusion machinery, including SNAP29 and NSF, which have been shown to function in autophagosome maturation or clearance (Schardt et al. 2009, Behrends et al. 2010). The interactome of RAB24 has not been conclusively determined but in their proteomic study of autophagy interaction network, Behrends et al. suggested that interactions of RAB24 with autophagic SNAREs, including SNAP29, likely occurred via NSF (Behrends et al. 2010). Although RAB24 is known to localize on autophagosomes, a direct interaction partner belonging to the autophagic machinery, if any, is yet to be identified. We and others have shown that RAB24 colocalizes with the autophagosome marker LC3 and that both of these proteins locate on the same autophagosome membranes. However, we have seen no evidence for interaction between RAB24 and LC3 or GABARAP with immunoprecipitation (our unpublished observation). Since RAB24 functions in the clearance of autophagic structures in nutrient rich conditions and colocalizes with SQSTM1 upon proteasome inhibition, we studied the possible interaction between RAB24 and SQSTM1 with an immunoprecipitation approach, but found no evidence for such an interaction.

Our findings are in agreement with the previously presented idea that RAB24 may act as a part of autophagic SNARE machinery. Our results further indicate that RAB24 is needed in autolysosome clearance after the degradation in these organelles is at least partially completed. One possible scenario would be that RAB24 works with NSF in the disassembly of the SNARE complexes after fusion with lysosomes, facilitating the recycling of the SNARE proteins and therefore enabling continuous rounds of fusion. Other possibilities might include a role in the release of digested material from autolysosomes to the cytosol, or involvement in budding of proto-lysosomes from

autolysosomes in ALR. The initiation of ALR can be observed in mammalian cells after some hours of starvation, when nutrients start to be released from autolysosomes. The released amino acids reactivate the autophagy repressor mTOR, which is a prerequisite for ALR (Yu et al. 2010). Interestingly, the timing of mTOR reactivation during starvation is similar to what we observed on the increase in colocalization of RAB24 and LC3. The colocalization increased after several hours of starvation, likely simultaneously with nutrient release from degrading autolysosomes.

It should be noted, however, that some simpler species, including the common research model organisms *Caenorhabditis elegans* and *Drosophila melanogaster*, lack the RAB24 gene, while it is found for example in zebrafish (*Danio rerio*), and in mammals. Therefore it is possible that there are functional redundancies with some other similar proteins. The role of RAB24 might also be specialized in a certain physiological state so that in certain species or cell types there is no need for it. One possibility could be that in higher organisms it is more important to recycle elements of the membrane fusion machinery if the need for continuing degradation persists, instead of degrading everything in lysosomes and producing more proteins to cover for the loss. These types of situations could be for instance prolonged starvation or a condition of persisting harmful autophagic cargo like aggregated or misfolded proteins. In this case there are likely differences among species in their ability to tolerate such conditions. These differences might stem from species-typical features such as lifespan. Whether RAB24 has such an effect at organismal level would be an interesting topic for a physiological study.

VI CONCLUSIONS AND FUTURE PERSPECTIVES

1. By using immunostaining at light as well as electron microscopy level and several high resolution electron microscopic methods, we described the distribution of autophagic compartments in NRK cells in relation to other organelles. We further showed that the phagophore and autophagosome can make physical contacts with several cellular compartments and that these can occur simultaneously with more than one organelle.
2. Electron tomography revealed direct membrane contacts between the phagophore and ER cisternae as well as ER exit sites, Golgi complexes, late endosomes or lysosomes and mitochondria. Autophagosomes were also occasionally adjacent to lipid droplets. ER was shown to be the most frequent proximal organelle to forming autophagosomes, and connections between phagophores and the ER were found in all constructed electron tomograms.

   While the discovery of membrane contact sites between these organelles is a significant advancement, in future studies it is essential to focus on the biological significance of these contacts and designate possible lipid transfer between the organelles. This could be explored for example through identifying lipid-transfer proteins or membrane fission/fusion machinery proteins that localize in these contact sites and by studying the possible roles of these proteins in the formation of autophagosomes.
3. With biochemical and microscopic methods we showed that RAB24 localizes to autophagosome membranes and that this localization is dependent on prenylation and GTP binding.

   In future experiments, RAB24 GTPase activity could be characterized in different conditions starting from pH, and the effect of the C-terminal histidines in this context should be tested with mutants. Also the effects of tyrosine phosphorylations on the GTPase activity and vice versa would be interesting to solve with mutant proteins.
4. We showed that RAB24 functions late in the autophagic process, after the formation of autolysosomes, most likely in their clearance.

   RAB24 functions most likely in the disassembly of the fusion machinery, recycling of the lysosomal membranes and enzymes in ALR, or in the release of the degraded material from autolysosomes. Further studies on the RAB24 interactome will likely provide more answers to questions about its mode of action. One starting point could be either the yeast two hybrid method, or affinity purification of protein complexes combined with mass spectrometry.

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